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# BRASSINOSTEROID-MEDIATED STRESS TOLERANCE: hormone pathways, genes and functions

(Spine title: **Brassinosteroid: Mechanism Of Stress Tolerance**)

(Thesis format: Integrated-article)

by

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Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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# ABSTRACT

Brassinosteroids (BRs) are naturally occurring plant steroid derivatives that play crucial roles in plant development and also promote tolerance to a range of abiotic stresses. Although much has been learned about their roles in plant development, the mechanisms by which BRs control plant stress responses and regulate stress-responsive gene expression are not fully known. It is also likely that the stress tolerance conferring ability of BRs is in part due to their interactions with other stress hormones. In the present study the stress tolerance effects of BR, interactions of BR with other plant hormones, and global genomic responses of BR in mediating stress tolerance were explored. Treatment with 24-epibrassinolide (EBR), a BR, enhanced dehydration tolerance of *Arabidopsis thaliana* (Arabidopsis), and this effect involved changes in the expression of dehydration-responsive genes. Study of EBR effects on the basic thermotolerance and salt tolerance of a collection of Arabidopsis mutants either deficient in or insensitive to abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and salicylic acid (SA), indicated that BR exerts anti-stress effects both independently as well as through interactions with other hormones. This study uncovered a critical role for NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1), a master regulator of SA-mediated defense responses, in BR-mediated increase in stress tolerance. Yet another finding was that ABA inhibits BR effects during stress, and that BR shares transcriptional targets with other hormones.

Whole-genome transcriptome analysis of BR-treated and untreated Arabidopsis seedlings under no-stress and heat stress revealed majority of the BR response genes to be related to stress tolerance, signal transduction and metabolism. Analysis of T-DNA insertion mutants of four BR response genes indicated that *WRKY17*, *WRKY33*, *ACP5* and *BRRLK* have stress-related functions.

As a final confirmation of a role for BR in stress tolerance, the *AtDWF4* gene encoding a BR biosynthesis enzyme was overexpressed in seeds of Arabidopsis. Preliminary studies of transgenic seedlings showed an increase in cold tolerance and the ability to overcome ABA-induced inhibition of germination.

In summary, the present study provides novel insights into the mechanism of BR-mediated stress tolerance by identifying genes and hormone interactions involved in this process.

KEY WORDS: Brassinosteroids, 24-epibrassinolide, heat stress, dehydration stress, salt stress, cold stress, Arabidopsis, cross-talk, microarray, *AtDWF4,* seed-specific overexpression.

## CO-AUTHORSHIP

**Chapter 3: Divi, U.K., Rahman, T., and Krishna, P.** (2009). Brassinosteroid-mediated abiotic stress tolerance in Arabidopsis involves interactions with abscisic acid, ethylene and salicylic acid pathways. BMC Plant Biol. (submitted)

Contributions of Co-authors:

T. Rahman

- Contributed data of abscisic acid mutant analysis

P. Krishna

- Chief advisor and investigator

## DEDICATION

To my parents and wife

## ACKNOWLEDGEMENTS

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Finally, I am forever indebted to my parents and my wife Mali for everything they have done so far and will continue to do. I owe a great deal to my brother Chitti Babu Divi for playing a major role in shaping up my career.

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## ABBREVIATIONS

| | |
|---|---|
| °C | degree Celsius |
| μE | microeinstein |
| μg | microgram |
| μM | micromolar |
| ABA | abscisic acid |
| ANOVA | analysis of variance |
| ARR | Arabidopsis response regulator |
| BAK | BRI associated kinase |
| BES | BRI ems suppressor |
| BIM | BES1-interacting Myc-like |
| BIN | Brassinosteroid insensitive |
| BKI | BRI1 kinase inhibitor |
| BL | brassinolide |
| bp | base pair |
| BR | brassinosteroid |
| BRF | brassinosteroid response transcription factor |
| BRI | Brassinosteroid insensitive |
| BSA | bovine serum albumin |
| BSU | BRI suppressor |
| BZR | brassinazole resistant |
| CAD | cinnmoyl alcohol dehydrogenase |
| CaMV | cauliflower mosaic virus |
| CBF | CRT/DRE Binding Factor |
| cDNA | complementary deoxyribonucleic acid |
| Col | columbia, accession of Arabidopsis |
| CPD | constitutive photomorphogenesis and dwarfism |
| cRNA | complimentary RNA |
| DET | deetiolated |
| DNA | deoxyribonucleic acid |

| | |
|---|---|
| dNTP | deoxynucleotide triphosphate |
| DRE | drought-responsive element |
| DWF | dwarf |
| EBR | 24-epibrassinolide |
| ET | ethylene |
| FDR | false discovery rate |
| FLS | flavone synthase |
| GO | gene ontology |
| GRP | glycine-rich protein |
| GSK | glycogen synthase kinase |
| GST | glutathione-s-transferase |
| GUS | β-glucuronidase |
| h | hour |
| HS | heat stress |
| HSF | heat shock factor |
| Hsps | heat shock proteins |
| JA | jasmonic acid |
| $Kan^R$ | kanamycin resistant |
| LEA | late embryogenesis abundant |
| LOX | lipoxygenase |
| LRR | leucine-rich repeat |
| LRR-RK | leucine-rich repeat receptor kinase |
| LTP | lipid transfer protein |
| $m^{-2}$ | per square meter |
| MAPK | mitogen activated protein kinase |
| min | minute |
| MIPS | Munich Information Centre for Protein Sequences |
| mRNA | messenger ribonucleic acid |
| MS | murashige and skoog medium |
| NaCl | sodium chloride |
| ng | nanogram |

| | |
|---|---|
| PR | pathogenesis-related |
| qRT-PCR | quantitative reverse transcription polymerase chain reaction |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| SA | salicylic acid |
| SAR | systematic acquired resistance |
| SDS | sodium dodecyl sulphate |
| SE | standard error |
| TAIR | The Arabidopsis Information Resource |
| TBARS | thiobarbituric acid reactive substances |
| T-DNA | transfer-deoxyribonucleic acid |
| UV | ultra violet |
| VC | vector control |
| vs | versus |
| WAK | wall associated kinase |
| WT | wild type |
| XTH | xyloglucan endotransglucosyltransferase/hydrolase |

# CHAPTER 1

# GENERAL INTRODUCTION

# CHAPTER 1

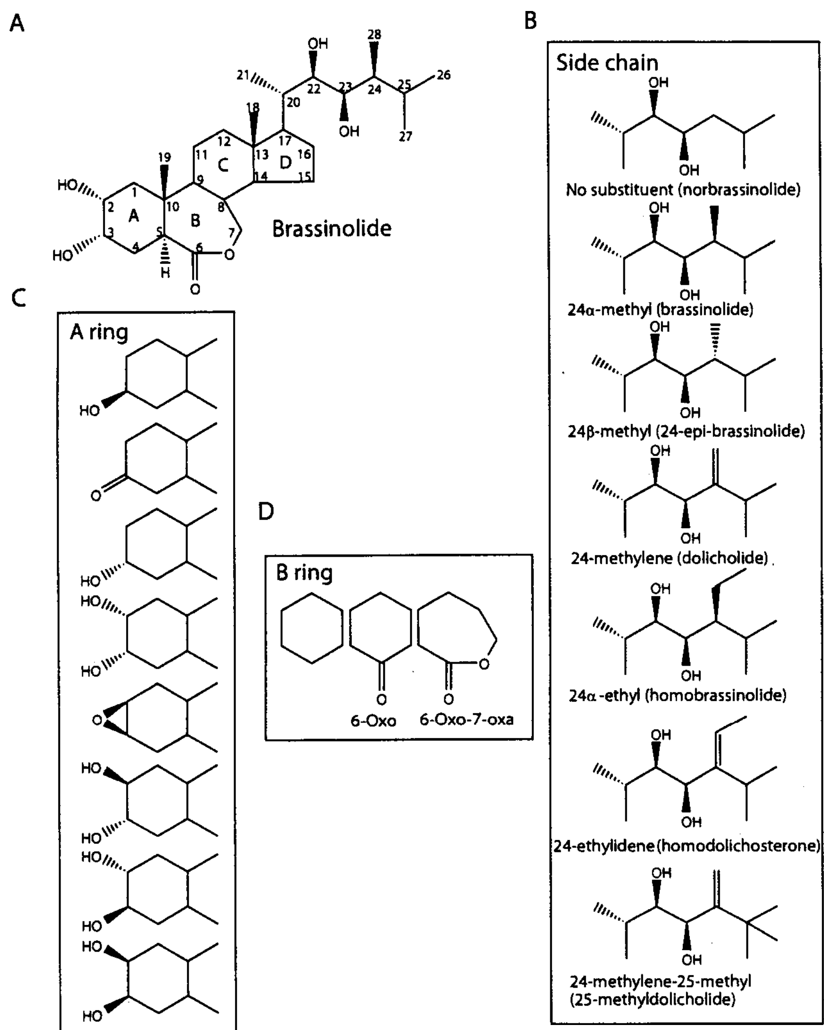
## 1.1 INTRODUCTION

### *1.1.1 Brassinosteroids: historical background*

The involvement of several plant hormones in the process of reproduction led plant scientists to believe that pollen could be a rich source of phytohormones. A search for novel plant hormones in pollen led to the discovery of 'Brassin' in rape (*Brassica napus*) pollen, which elicited strong growth-promoting activity in the bean second internode assay (Mitchell et al., 1970). Later, in a major coordinated effort involving several United States Department of Agriculture (USDA) laboratories, the active component of Brassin was isolated in minute quantities (4 mg from 227 kg of bee-collected rape pollen). X-ray crystallographic studies identified the substance to be a steroidal lactone, which was named as brassinolide (BL) (Grove et al., 1979). BL is the first characterized brassinosteroid (BR) and since then about 60 additional BRs have been identified from various plant species, indicating that BRs are ubiquitous in the plant kingdom (Bajguz and Tretyn, 2003). Although BRs are found in all plant organs, their endogenous levels are quite low. Pollen and immature seeds are the richest sources of BRs with levels ranging between 1-100 ng $g^{-1}$ fresh weight. Shoots and leaves usually have lower amounts in the range of 0.01-0.1 ng $g^{-1}$ fresh weight (Bajguz and Tretyn, 2003).

The structure of BL, the most active BR, is shown in Figure 1.1A. BRs show structural variations and fall into seven categories depending on the side chain structure derived from their parent sterols (Figure 1.1B). There are also differences in the number of hydroxyl groups with varying configurations at the C-2 and C-3 positions in the A ring and the presence of either a ketone or a lactone moiety at C-6 and C-7 in the B ring (Figure 1.1C and D). Certain structural features are common to all active BRs. The A and B rings are always in *trans* configuration, which is determined by an α hydrogen at C-5; the B ring contains a 6-oxo or a 6-oxo-7-oxa group (Figure 1.1D); and the hydroxyl groups at C-2 and C-3 are *cis* oriented (Choe, 2004). These structural features are deemed to be essential for the activity of BRs. The growth-promoting activities of BR were first noted by exogenous application of BRs at nanomolar to micromolar concentrations to

**Figure 1.1** Structural variations in brassinosteroids. **A)** Structure of brassinolide. **B)** side chain structural variations. **C)** variations in the A ring. **D)** variations in the B ring (Choe, 2004).


A
Brassinolide
B
Side chain
No substituent (norbrassinolide)
24α-methyl (brassinolide)
24β-methyl (24-epi-brassinolide)
24-methylene (dolicholide)
24α-ethyl (homobrassinolide)
24-ethylidene (homodolichosterone)
24-methylene-25-methyl
(25-methyldolicholide)
C
A ring
D
B ring
6-Oxo
6-Oxo-7-oxa

plants, which induced a wide spectrum of physiological effects such as cell elongation, vascular differentiation, ethylene biosynthesis, and retardation of abscission (Mandava, 1988). Studies of BR-deficient and BR-insensitive Arabidopsis mutants in the mid 1990s provided convincing evidence for an essential role of BRs in plant development (Clouse and Sasse, 1998; Clouse, 2002). For this reason BRs now have the status of phytohormone.

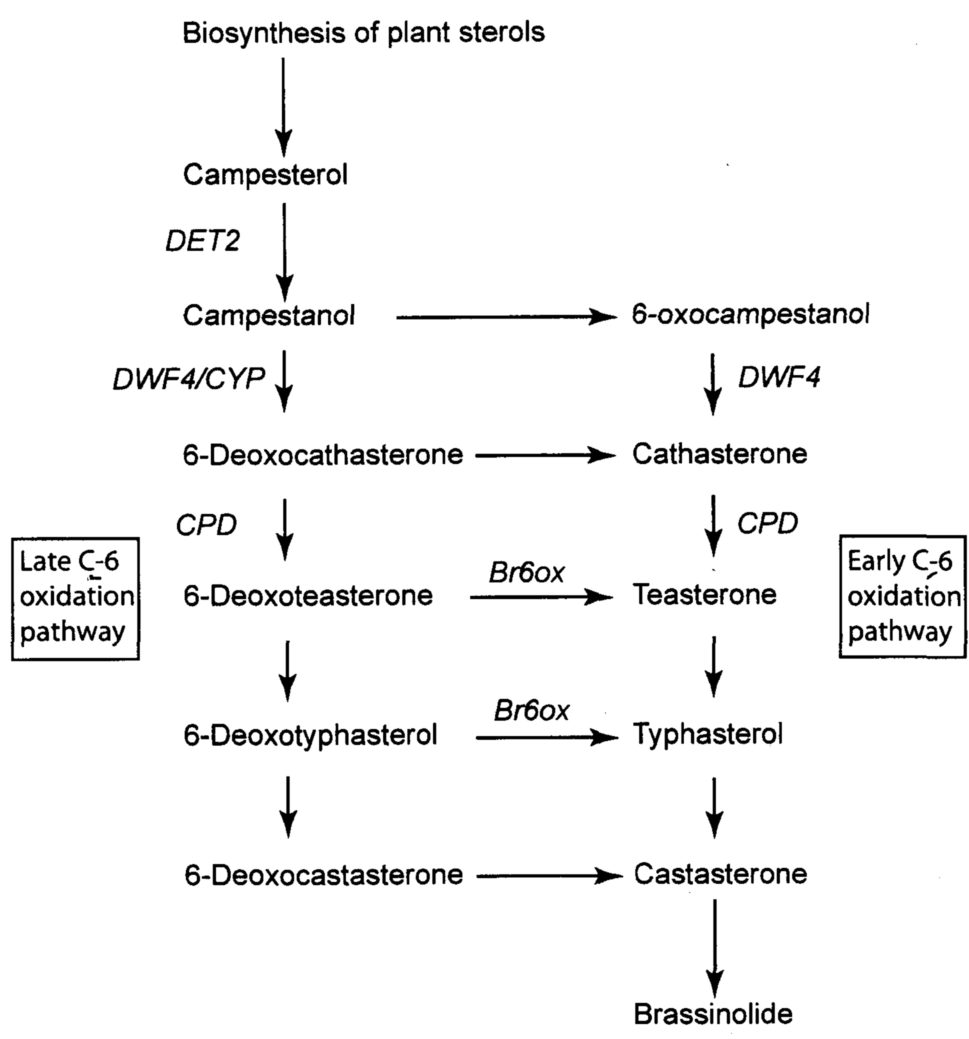
### *1.1.2 Brassinosteroid biosynthesis*

The BR biosynthetic steps have been elucidated by feeding cell suspension cultures of *Catharanthus roseus* with isotope-labeled BR biosynthetic intermediates and by genetic analysis of BR-deficient mutants (Fujioka et al., 1995; Bishop and Yokota, 2001; Fujioka and Yokota, 2003). BRs are derived from steroids and the pathway leading to their biosynthesis can be divided into a sterol-specific pathway and a BR-specific pathway. In the sterol specific pathway, squalene synthesized from mevalonic acid (MVA) is converted to two major plant sterols, sitosterol and campesterol. Campesterol is the precursor of $C_{28}$ BRs and is initially converted to campestanol. Stepwise metabolic experiments have revealed the presence of two parallel pathways from campestanol to castasterone termed as early and late C-6 oxidation pathways (Figure 1.2; Noguchi et al., 2000). Another branching pathway termed as the early C-22 oxidation pathway (Fujioka et al., 2002), as well as a shortcut pathway from campesterol to 6-deoxytyphasterol involving C-23 oxidation, have also been described (Ohnishi et al., 2006). In the final steps, castasterone is converted to BL by lactonization of the B ring.

The isolation and characterization of dwarf mutants that are defective in BR biosynthesis was also instrumental in understanding the BR biosynthetic pathway. The Arabidopsis BR-deficient mutants are characterized by short robust stature, dark-green, round and curly leaves, reduced fertility and prolonged life span (Kwon and Choe, 2005). In addition, when grown in the dark, severe BR dwarfs often display abnormal etiolation patterns including reduction in hypocotyl length, open cotyledons, and absence of apical hook (Choe et al., 2000). Exogenous application of BR can recover BR-deficient mutants to the wild-type (WT) phenotype (Szekeres et al., 1996; Clouse and Sasse, 1998). Several genes encoding BR biosynthetic enzymes have been characterized using BR biosynthesis

mutants of Arabidopsis, pea, tomato and rice (Kwon and Choe, 2005). The dwarf mutant *de-etiolated2* (*det2*) was identified in a study of light-regulated development in Arabidopsis (Chory et al., 1991). The *DET2* gene encodes a steroid 5α-reductase, which is involved in the conversion of 22-OH-4-en-3-one to 22-OH-3-one (Noguchi et al., 1999). The oxidation steps of BR biosynthesis are catalyzed by cytochrome P450 monooxygenases. The C-22 and C-23 hydroxylation reactions are mediated by the P450s DWF4 (DWARF4) and CPD (CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM), respectively (Choe et al., 1998; Szekeres et al., 1996), and the C-6 oxidation is catalyzed by BR-6-oxidases (BR6ox) (Figure 1.2; Shimada et al., 2001). The C-22 hydroxylation step catalyzed by DWF4 is considered a rate-determining step in the BR biosynthetic pathways. The *DWF4* gene encodes a cytochrome P450 monooxygenase (CYP90B1) that catalyzes conversion of cathasterone to 6-deoxocathasterone in the late C-6 oxidation pathway and of 6-oxocampestanol to cathasterone in the early C-6 oxidation pathway (Figure 1.2; Choe et al., 1998). The Arabidopsis *dwf4* mutants are severely dwarf displaying typical BR-deficient phenotypes (Choe et al,. 1998). The C-23 hydroxylation is catalyzed by CPD, another cytochrome P450 enzyme belonging to the CYP90 family (CYP90A1). The *cpd* mutant is an extreme dwarf and can be rescued only by 23α-hydroxylated BRs, indicating that CPD acts as a C-23 steroid hydroxylase (Szekeres et al., 1996). A genetic defect similar to *cpd* has also been found in the tomato *dpy (dumpy)* mutant, which can also be rescued only by C-23 hydroxylated BRs (Koka et al., 2000). Two other members of the CYP90 family, CYP90C1/ROTUNDIFOLIA3 and CYP90D1, act as functionally redundant C-23 hydroxylases in BR biosynthesis (Ohnishi et al., 2006). The gene responsible for C-6 oxidation was first identified in tomato. Analysis of the tomato *DWARF* gene, whose encoded product shares high amino acid similarity with mammalian steroid hydroxylases, revealed that the C-6 oxidation of 6-deoxoBRs to 6-oxoBRs is mediated by the cytochrome P450, CYP85 (Bishop et al., 1999). The Arabidopsis genome has two copies of the tomato *DWARF* homologs: *AtBR6ox1 (CYP85A1)* and *AtBR6ox2 (CYP85A2)*. Shimada et al. (2003) showed that both genes converted 6-deoxocathasterone, 6-deoxoteasterone, 6-deoxotyphasterol and 6-deoxocastasterone to their respective 6-oxidized forms (Figure 1.2).

**Figure 1.2** A simplified illustration of the BR biosynthesis pathway (Divi and Krishna, 2009b).



Biosynthesis of plant sterols
Campesterol
DET2
Campestanol
6-oxocampestanol
DWF4/CYP
DWF4
6-Deoxocathasterone
Cathasterone
CPD
CPD
Late C-6 oxidation pathway
Early C-6 oxidation pathway
6-Deoxoteasterone
Br6ox
Teasterone
6-Deoxotyphasterol
Br6ox
Typhasterol
6-Deoxocastasterone
Castasterone
Brassinolide

The Arabidopsis BR biosynthetic P450 genes are under negative feedback regulation by BRs and require BR signaling for this auto-regulation (Bancos et al., 2002; Tanaka et al., 2005). Thus, the C-22 and C-23 hydroxylation and the C-6 oxidation reactions are key regulatory steps in BR biosynthesis, and hence the P450 enzymes catalyzing these reactions are potential targets for biotechnological intervention to modulate endogenous BR levels for enhanced plant traits.

### *1.1.3 Brassinosteroid signaling*

A current model of BR signaling is illustrated in Figure 1.3. Unlike animal steroids that are perceived by nuclear receptors, BRs are perceived at the cell surface by BRI1 (BRASSINOSTEROID-INSENSITIVE 1), a plasma membrane localized leucine-rich repeat receptor-like kinase (LRR-RLK) (He et al., 2000). BR binding to BRI1 induces a series of biochemical events, such as autophosphorylation of BRI1 in its C-terminal domain, dissociation of an inhibitory protein, BKI1 (BRI1 KINASE INHIBITOR 1), and association of BRI1 with another LRR-RLK, BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) (Nam and Li, 2002; Wang and Chory, 2006), which likely enhances signaling output through reciprocal BRI1 transphosphorylation (Wang et al., 2008). Other known components of the BR signaling pathway include the glycogen synthase kinase-3, BIN2 (BRASSINOSTEROID-INSENSITIVE 2), which negatively regulates the transcription factors BZR1 (BRASSINAZOLE-RESISTANT 1) and BES1 (*bri1*-EMS-SUPPRESSOR 1) by phosphorylating them (Li and Nam, 2002; Vert and Chory, 2006), while the phosphatase BSU1 (*bri1* SUPPRESSOR 1) positively regulates BR signaling possibly by dephosphorylating BZR1 and BES1 (Mora-Garcia et al., 2004). BIN2-catalyzed phosphorylation likely inhibits BZR1 and BES1 functions through several modes, such as targeted degradation, reduced DNA binding, and cytoplasmic retention through interaction with 14-3-3 proteins (Gampala et al., 2007). In summary, BRI1 binding to BR inactivates BIN2 and activates BSU1, resulting in the activation and nuclear accumulation of BZR1 and BES1. Activated BZR1 and BES1 directly bind the promoters of BR-regulated genes to affect their expression (He et al., 2005; Yin et al., 2005). Neither BIN2 nor BSU1 has been shown to interact with BRI1; thus, how BR signal is transmitted to these downstream proteins is currently not known.

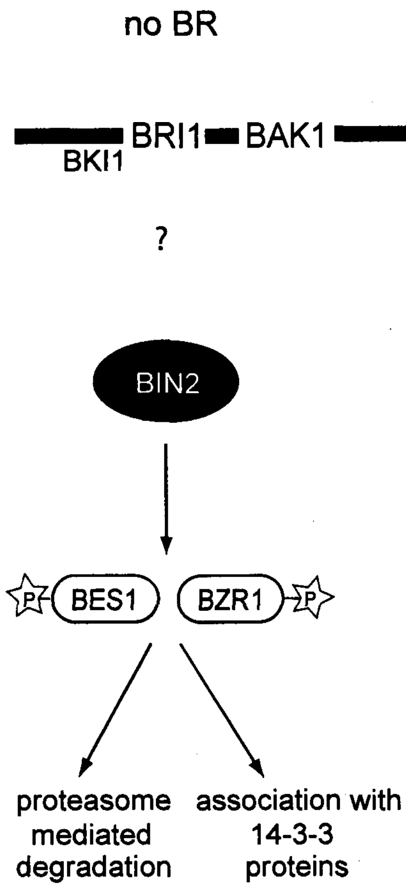
The recently identified BR signaling kinase BSK3 functions downstream of BRI1 (Tang et al., 2008), but it remains to be seen if BIN2 is a direct downstream target of BSK3 or its relatives.

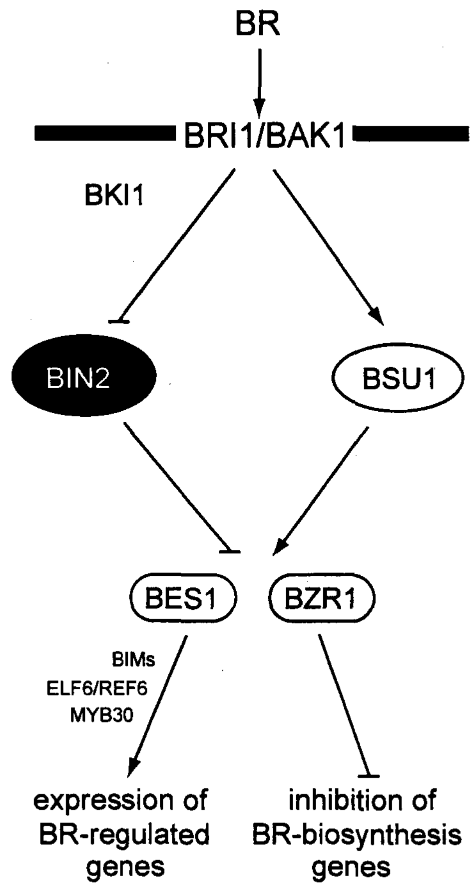
Numerous BR-regulated genes have been identified by genome wide microarray analyses (Goda et al., 2002; Müssig et al., 2002; Vert et al., 2005). The majority of the known BR-regulated genes are associated with plant growth and development processes, such as cell wall modification, cytoskeleton formation, and hormone synthesis (Vert et al., 2005). The modes of action of BZR1 and BES1 are currently known for only a limited set of BR-responsive genes. BZR1 binds to the CGTG(T/C)G motif found in the promoters of BR biosynthetic genes, *CPD* and *DWF4,* to suppress their expression (He et al., 2005), while BES1 binds to the CANNTG motif (E-box) in the *SAUR-AC1* promoter to activate gene expression (Yin et al., 2005). In view of the number of physiological processes regulated by BR, it was hypothesized that BZR1 and BES1 heterodimerize with other transcriptional factors to regulate transcriptional processes. Indeed, BES1 has been demonstrated to interact with BIMs (BES1-INTERACTING MYC-LIKE PROTEINS), leading to enhanced binding of BES1 to the *SAUR-AC1* promoter (Yin et al., 2005), MYB30 (MYB DOMAIN PROTEIN 30), a transcription factor that acts as a positive regulator of the hypersensitive cell-death response (Li et al., 2009), and the jumonji (Jmj) domain-containing proteins ELF6 (EARLY FLOWERING 6) and REF6 (RELATIVE OF EARLY FLOWERING 6) that are involved in regulating flowering time (Yu et al., 2008). Since Jmj domain-containing histone demethylases are involved in many developmental processes and diseases, it is possible that recruitment of these proteins by BES1 is one of the ways by which BR affects diverse biological processes. Similarly, MYB30 and BES1 bind to a conserved MYB-binding site and E-box sequences, respectively, in the promoters of genes that are regulated by both BRs and AtMYB30, promoting the expression of a subset of BR target genes (Li et al., 2009).

### *1.1.4 Brassinosteroid-regulated genes*

The best characterized direct BR effects are the early transcriptional changes in response to BR treatment (Müssig et al., 2002; Müssig and Altmann, 2003; Vert et al.,

**Figure 1.3** A current model for BR signaling pathway. **A)** no BR. **B)** in the presence of BR signal (Divi and Krishna, 2009b).


A
no BR
BKI1
BRI1
BAK1
?
BIN2
P
BES1
BZR1
P
proteasome mediated degradation
association with 14-3-3 proteins


B
BR
BRI1/BAK1
BKI1
BIN2
BSU1
BES1
BZR1
BIMs
ELF6/REF6
MYB30
expression of BR-regulated genes
inhibition of BR-biosynthesis genes

2005). BR-regulated genes encode enzymes and proteins involved in diverse physiological processes including cell wall loosening (Müssig and Altmann, 2003), regulation of cell division (Hu et al., 2000), carbohydrate metabolism (Goetz et al., 2000), ethylene (ET) (Yi et al., 1999) and jasmonic acid (JA) biosynthesis (Müssig et al., 2000), protein translation (Jiang and Clouse, 2001; Dhaubhadel et al., 2002), and other physiologic processes (Müssig et al., 2006). Cell wall modifying enzymes, such as xyloglucan endotransglucosylase/hydrolases (XTHs) and expansins were among the first to be identified as BR-regulated. XTHs are known to be involved in cell wall loosening to facilitate wall expansion and in biogenesis of cell wall material (Campbell and Braam, 1999; Rose et al., 2002). The transcript levels of several XTHs from different plant species, such as *BRU1* from *Glycine max* (Zurek and Clouse, 1994), *TCH4* from Arabidopsis (Xu et al., 1995), *LeBRI1* from *Lycopersicon esculentum* (Koka et al., 2000), and *OsXTR1* and *OsXTR3* from *Oryza sativa* (Uozu et al., 2000), were found to be up-regulated by BR treatment. *CYCD3 (CYCLIN D3)*, a gene involved in the regulation of cell division, is another BR-induced gene (Hu et al., 2000). In tomato, *SUS4 (SUCROSE SYNTHASE 4)* transcripts accumulate throughout the meristem following BR treatment, indicating a role for BRs in carbon metabolism (Pien et al., 2001). The expression of β-tubulin gene, *TUB1*, is reduced in *dim (diminuto/dwarf1)*, a BR-deficient mutant in rice (Takahashi et al., 1995). Accordingly, the expression of a β-tubulin gene of *Cicer arietinum* is induced by exogenous BRs (Munoz et al., 1998), indicating a role for BRs in microtubule formation.

In addition to the small scale studies on BR genomic responses, several recent studies have identified genome wide BR responses by employing DNA microarrays (Goda et al., 2002; Müssig et al., 2002; Yin et al., 2002; Vert et al., 2005). All studies thus far have focused on the effects of short-term BR treatment of plants and an important outcome of these studies is that BR responses are modest by nature. About 80% of genes consistently detected in these studies showed estimated expression changes of < 2-fold (Vert et al., 2005). BR-regulated genes consist of those that are specifically regulated by BRs, as well as those that show regulation in response to additional phytohormones, such as auxins, gibberellic acid (GA), ethylene (ET) and jasmonic acid (JA), indicating cross-talk of BR with other plant hormones.

### *1.1.5 Interaction of Brassinosteroids with other phytohormones*

BRs interact with other plant hormones in mediating developmental responses (Khripach et al., 1999). Experimental evidence points to interactions of BR with auxin (Mouchel et al., 2006; Hardtke et al., 2007), GA (Bouquin et al., 2001; Shimada et al., 2006), abscisic acid (ABA) (Steber and McCourt, 2001; Abrahám et al., 2003), ET (Yi et al., 1999; Arteca and Arteca., 2001) and JA (Kitanaga et al., 2006; Müssig et al., 2006), primarily in plant growth regulatory processes. With the exception of BR-auxin interaction, little is known in terms of genes how BR interacts with other hormones. Recent progress made towards understanding BR-auxin interaction can serve as a paradigm for how two hormones could interact at multi-levels. Auxin and BR share a number of target genes, many of which are involved in growth related processes (Hardtke et al., 2007). Since promoter regions in BR-responsive genes are enriched in Auxin Response Factor (ARF)-binding sites, and binding sites of BES1 are over-represented in genes regulated by both hormones, regulatory elements in gene promoters represent a point of cross-talk between auxin and BR (Nemhauser et al., 2004). Recently, the BR-regulated BIN2 kinase was demonstrated to phosphorylate ARF2, a member of the ARF family of transcriptional regulators, leading to loss of ARF2 DNA binding and repression activities (Vert et al., 2008). Thus, in this model ARF2 links BR and auxin signaling pathways. In addition to gene coregulation, BR can also promote auxin transport (Li et al., 2005), and optimal auxin action is dependent on BR levels (Mouchel et al., 2006).

The role of BR in plant responses to abiotic stress has become well established over the last decade (Divi and Krishna, 2009a), but there are very few reports indicating how BR interacts with other stress related hormones and their signaling pathways in conferring stress tolerance. While there exists evidence to indicate that BR increases ET and JA levels under normal growth conditions (Arteca and Arteca, 2001; Kitanaga et al., 2006), there appears to be only one report linking BR with increase in ABA levels in the lower plant *Chlorella vulgaris* under stress condition (Bajguz, 2009). Very recently it has been demonstrated that ABA inhibits BR signaling through phosphorylation of BES1 (Zhang et al., 2009). Currently there are no studies at the genetic level as to how BR interacts with other hormones under stress conditions.

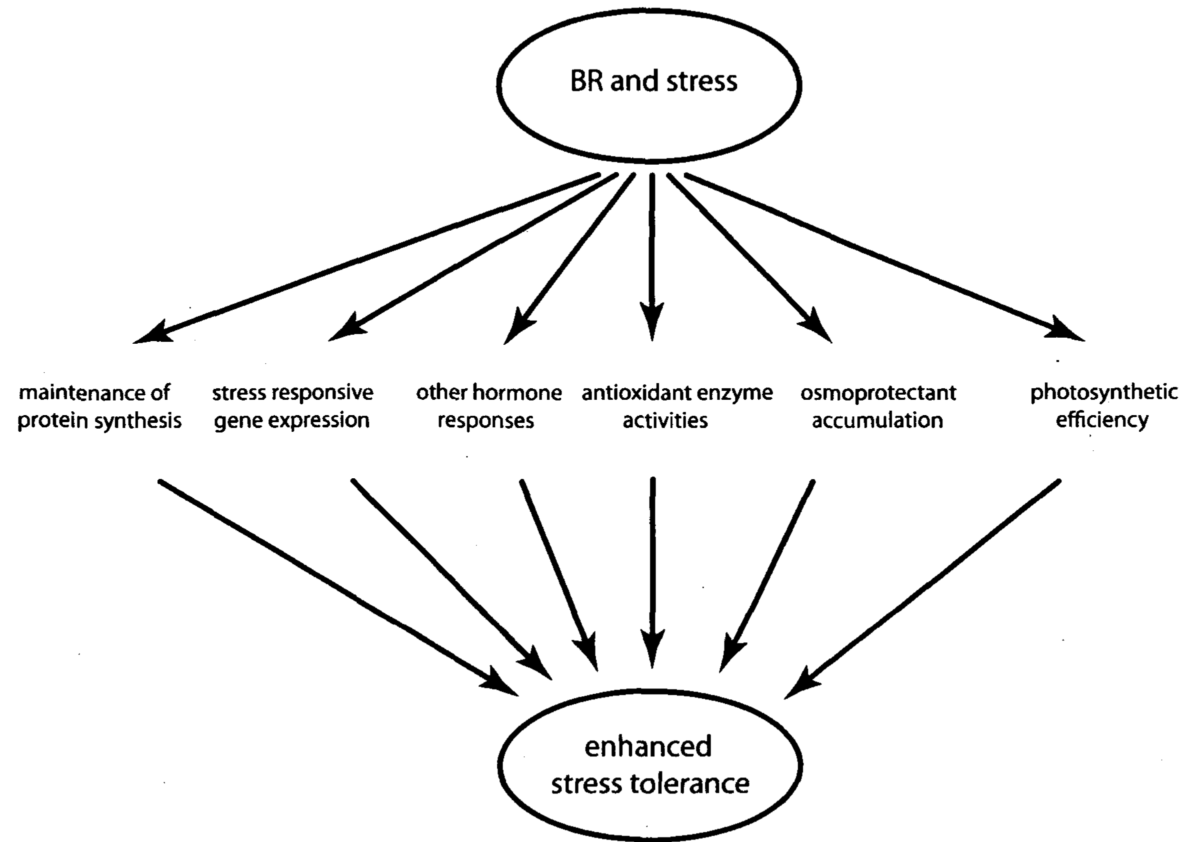
### *1.1.6 Brassinosteroid-mediated stress tolerance*

Similar to the growth promoting effects of BR, the stress protective properties of BRs had also been noticed prior to the time research on BR caught the interest of the larger plant community. However, these preliminary studies manifested considerable variability in the efficacy of BRs to promote stress tolerance, which likely contributed to the relatively slower pace of progress made in this direction. It is believed that the mode of BR application (seed soak, root soak or foliar spray), as well as the developmental stages at which BR was applied, were the primary reasons for the inconsistency in the results of earlier studies (Krishna, 2003; Divi and Krishna, 2009a). Recent attempts to study BR effects on plant stress responses under standardized conditions have indeed yielded reproducible effects. These studies have largely contributed towards understanding BR effects on diverse processes that converge into producing enhanced tolerance to a broad range of stresses (Figure 1.4; Divi and Krishna, 2009a).

**TEMPERATURE STRESS**

The effects of BR on plant's ability to cope with high and low temperatures have been evaluated in several studies. Positive consequences of BRs in combating chilling stress were reported in maize, cucumber, tomato and rice (He et al., 1991; Katsumi, 1991; Kamuro and Takatsuto, 1991). A general conclusion that can be derived from these studies is that the BR effects on growth and yield are more pronounced during cold stress conditions than under normal conditions. More recently, the regulatory relationship between BR and chilling was investigated in a proteomic study using mung bean epicotyls. Treatment with 24-epibrassinolde (EBR), a BR, could partly recover the elongation of mung bean epicotyls after initial suppression of growth by chilling conditions (Huang et al., 2006). Concomitantly, 17 proteins observed to be down-regulated by chilling stress were up-regulated by EBR. These up-regulated proteins were functionally linked with methionine assimilation, ATP synthesis, cell wall construction, and stress response (Huang et al., 2006).

**Figure 1.4** BR promotes stress tolerance by potentiating diverse cellular processes involved in stress tolerance (Divi and Krsihna, 2009a).


BR and stress
maintenance of protein synthesis
stress responsive gene expression
other hormone responses
antioxidant enzyme activities
osmoprotectant accumulation
photosynthetic efficiency
enhanced stress tolerance

The positive effects of BR under high temperature conditions were noted in tomato, as better photosynthetic efficiency, *in vitro* pollen germination and pollen tube growth, and reduction in pollen bursting as compared to untreated plants. These effects correlated with higher accumulation of mitochondrial small heat shock proteins (Singh and Shono, 2005), reduction in total hydrogen peroxide and malonaldehyde contents, and increases in the activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPOD), and catalase (CAT) in BR-treated tomato plants (Ogweno et al., 2007). This BR-mediated increase in carboxylation efficiency and antioxidant enzyme activities must contribute to mitigating the detrimental effects of high temperatures on plant growth.

**SALT STRESS**

Salinity stress inhibits seed germination and plant growth. BR reduced the inhibitory effects of salt on seed germination in *Eucalyptus camaldulensis* (Sasse et al., 1995), rice (Anuradha and Rao, 2001), and *B. napus* (Kagale et al., 2007). In salt sensitive IR-28 rice, EBR reduced the extent of oxidative damage incurred by salt stress, which correlated with less lipid peroxidation, significant increase in the activity of APX, higher soluble protein content and higher accumulation of the protective osmolyte proline (Ozdemir et al., 2004).

**DROUGHT STRESS**

Measurements of both growth and physiological parameters suggest that BRs diminish the negative effects of water stress. Soaking the roots of *Robinia pseudoacacia* L. seedlings in brassinolide (BL) prior to planting increased the survival and growth of seedlings under simulated drought conditions (Li et al., 2007). BL-treated seedlings accumulated higher levels of osmolytes like proline and soluble sugars, had higher leaf water content and greater increases in the activities of antioxidant enzymes SOD, peroxidase (POD) and CAT as compared to untreated seedlings. Also, the stress-induced reduction in the transpiration rate, stomatal conductance and malondialdehyde content was less severe in BL-treated seedlings as compared to untreated seedlings (Li et al.,

2007). A drought susceptible variety of French bean was less affected in nodule number, nodulated root mass and root length under drought stress in response to EBR treatment (Upreti and Murti, 2004).

## PATHOGEN ATTACK

The potential of BR to induce resistance to a broad range of pathogens, including fungus, bacteria and virus, was demonstrated in tobacco and rice (Nakashita et al., 2003). Tobacco plants treated with BL exhibited enhanced resistance to the viral pathogen tobacco mosaic virus (TMV), the bacterial pathogen *Pseudomonas syringae*, and the fungal pathogen *Oidium* sp. Similarly, BL-treated rice plants were resistant against rice blast and bacterial blight caused by *Magnaporthe oryzae* and *Xanthomonas oryzae,* respectively. Application of brassinazole (Brz), a BR biosynthesis inhibitor, had a suppressive effect in a dose dependent manner on the defense response of tobacco against TMV, indicating that endogenous BR is involved in disease resistance (Nakashita et al., 2003). Interestingly, in this study BL treatment had no affect on endogenous salicylic acid (SA) levels or the expression of pathogenesis-related (*PR*) genes, suggesting that BR-mediated disease resistance is distinct from the SA-induced systemic acquired resistance (SAR). In contrast to these findings, higher *PR* gene expression in response to BR treatment had been noted in a few studies. The BR-deficient mutant *cpd* showed remarkably lower expression of *PR-1*, *PR-2*, and *PR-5* as compared to WT, but overexpression of the *CPD* cDNA resulted in a significant induction of these genes in the complemented lines (Szekeres et al., 1996). Short-term treatment (1 h and 6 h) of Arabidopsis seedlings with BL also induced the expression of *PR-1* and *PR-2* genes (Seo et al., 2008). These results together make a case for *PR* genes to be primary response genes of BR. Surely then, the mechanism by which BR induces their expression warrants exploration in the future.

## OTHER STRESSES

The involvement of BR in signaling events during UV-B stress (280-315 nm) was investigated in Arabidopsis BR-deficient (*det2, dim1, cpd*) and BR-insensitive (*bri1*) mutants (Sävenstrand et al., 2004). These mutants showed reduced expression of a set of UV-B responsive defense genes [chalcone synthase (*CHS*), *PYROA*, *PR-5*, and a gene

regulated by very low levels of UV-B, *MEB5.2*] as compared to WT. Interestingly, the expression of *PR-5* transcripts was most affected by BR-deficiency. Overall, these results indicate that a complete BR pathway is required for proper UV-B dependent gene expression in Arabidopsis (Sävenstrand et al., 2004).

A few studies have demonstrated that BR treatment can protect against the toxic effects of heavy metals (Hasan et al., 2008), as well as block the uptake and accumulation of heavy metals (Sharma and Bhardwaj, 2007). While earlier studies suggested that BRs can protect plants from chemical damage caused by pesticides and insecticides (Cutler, 1991) these effects have not been substantiated in recent studies.

## GENETIC EVIDENCE FOR THE ROLE OF BR IN PLANT STRESS RESPONSES

Although numerous studies described so far have suggested a role for BR in plant stress responses, only a few genetic studies have been conducted to confirm this property. The most convincing evidence comes from the analysis of knockout (KO) mutants of *OsGSK1*, the rice ortholog of *BIN2,* a negative regulator of BR signaling (Koh et al., 2007). The *OsGSK1* KO mutants obtained by T-DNA insertion showed enhanced tolerance to cold, heat, salt, and drought stresses when compared with non-transgenic (NT) segregants. For example, the wilting ratios for KO mutants were about 20, 26 and 36% lower as compared with NT plants after cold, heat, and salt stress, respectively. Furthermore, higher expression of abiotic stress-responsive genes was observed in the KO plants under different abiotic stress conditions. The strong likeness of these results with those obtained in the systemic studies carried out in our laboratory using BR-treated *B. napus* and Arabidopsis (Dhaubhadel et al., 1999; Kagale et al., 2007), strongly suggest that BR has roles in plant stress responses.

## SYSTEMATIC STUDY TO DISSECT THE ROLE OF BR IN ABIOTIC STRESS TOLERANCE

To systematically dissect the mechanisms involved in BR-mediated tolerance to abiotic stresses, first it was established that BR treatment could consistently increase the freezing, heat and drought tolerance of plants (Wilen et al., 1995; Dhaubhadel et al., 1999; Kagale et al., 2007). Next, the effect of BR on the expression of prototype genes

up-regulated in response to heat, cold and drought stress were studied in *B. napus* and Arabidopsis. Interestingly, while BR-treated *B. napus* seedlings accumulated significantly higher levels of heat shock proteins (hsps) in response to heat stress (HS) (Dhaubhadel et al., 1999), there was little to no difference in hsp levels in BR-treated Arabidopsis seedlings as compared to untreated seedlings (Kagale et al., 2007). In response to cold stress, transcript levels of structural genes involved in cold and dehydration tolerance (*RD29A*, an ortholog of *BN115* and *COR47*) were observed at much higher levels in BR-treated Arabidopsis seedlings as compared to untreated seedlings. By contrast, there were no noticeable differences between untreated and treated *B. napus* seedlings in the expression of regulatory genes *BNCBF5* and *BNDREB*, and structural genes *BN115, BN28*, and *hsp90* (Kagale et al., 2007). While these results indicate that BR-mediated stress-responsive gene expression profiles are somewhat different in *B. napus* and Arabidopsis, it is also clear that, in general, there is higher induction of stress-responsive genes in BR-treated *vs.* untreated seedlings.

Further investigation of why BR-treated *B. napus* seedlings accumulate higher levels of hsps indicated that BR treatment both limits the loss of the components of the translational apparatus during heat stress, and increases their levels during recovery, which correlate with higher hsp synthesis during stress, more rapid resumption of cellular protein synthesis following HS and a higher survival rate (Dhaubhadel et al., 2002). To identify additional BR-induced gene expression changes in *B. napus* seedlings, the differential display–reverse transcription PCR technique was used. Substantial changes were identified in the expression levels of genes encoding a mitochondrial transcription termination factor (mTERF)-related protein, glycine-rich protein 22 (GRP22), myrosinase, and 3-ketoacyl-CoA thiolase (Dhaubhadel and Krishna, 2008). Transcripts of mTERF-related protein, *GRP22*, and myrosinase were present at approximately 2-, 4-, and 6-fold higher levels, respectively, in treated seedlings before HS, whereas those of 3-ketoacyl-CoA thiolase rose to higher levels in treated seedlings during exposure to HS. These results indicate that BR treatment in *B. napus* leads to substantial changes in the expression levels of genes involved in a variety of physiologic responses, either before or during stress exposure.

### *1.1.7 Objectives and significance of the present research*

The potential of BRs to increase plant resistance to a range of stresses has been established in several studies. BR has also been shown to induce the expression of stress-responsive genes, however, our understanding of the mechanisms by which BR induces stress tolerance is still in its infancy. A systematic and thorough investigation of the molecular mechanism of BR-mediated stress tolerance in model plant systems is important from the standpoint of basic research as well as commercial applications of BR to agriculture.

The objectives of the present study are:

1. to study the effects of exogenous application of BR on dehydration stress in Arabidopsis and to establish conditions under which these effects can be studied in a reproducible manner at the morphological and molecular level,
2. to study the interactions of BR with other plant hormones in mediating stress responses by using various hormone biosynthesis and signaling mutants of Arabidopsis,
3. to identify genes differentially expressed in BR-treated *vs.* untreated Arabidopsis seedlings under no-stress and HS conditions by use of DNA microarrays, and to carry out knockout mutant analysis on a subset of genes for stress-related functions, and
4. to overexpress a BR biosynthetic enzyme AtDWF4 in Arabidopsis seeds and analyze the resulting transgenic plants for stress tolerance.

The results of the present study will shed light on a macro scale on how BR promotes stress tolerance in plants, as well as how BR-mediated regulation of stress responses is integrated with its effects on plant growth and development. Such an understanding is important when contemplating changes of plant architecture, productivity or sustainability through manipulation of BR levels.

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# CHAPTER 2

## Brassinosteroid confers dehydration tolerance in *Arabidopsis thaliana*

**A version of this chapter has been published as part of the following article.**

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# CHAPTER 2

## 2.1 INTRODUCTION

Brassinosteroids (BRs) are a class of plant steroidal compounds that are essential for normal growth and development of plants. BRs regulate a wide range of biological processes, such as cell division and expansion, xylem differentiation, seed germination, vegetative growth and apical dominance (Sasse, 2003). Although the growth promoting properties of BRs were known in the early 1970s, the evidence for an essential role for BRs in plant development came through the isolation and characterization of BR-deficient and BR-insensitive mutants. BR-deficiency in biosynthetic mutants leads to phenotypic alterations such as dwarfism, small dark-green leaves, delayed flowering, senescence and reduced fertility, all of which can be rescued by exogenous treatment with BR in a dose dependent manner (Kwon and Choe, 2005). These observations indicate that BR functions as a hormone, and indeed, BR is now considered a plant hormone.

In addition to its role in plant growth and development, BR has been linked with protection of plants against a variety of environmental stresses, including high and low temperature stress, drought, salinity, herbicidal injury, and pathogen attack (Khripach et al., 2000; Krishna, 2003). However, studies confirming the ability of BR to modulate plant stress responses, as well as providing a framework under which such effects of BR can be studied in a reproducible manner are lacking. Previous studies in the lab showed that treatment of *Brassica napus* and tomato seedlings with 24-epibrassinolide (EBR), a BR, increased the basic thermotolerance of seedlings and led to higher accumulation of the major classes of heat shock proteins (hsps) as compared to untreated seedlings (Dhaubhadel et al., 1999). Further investigation into how hsps accumulate to higher levels in EBR-treated seedlings revealed that EBR modulates the translational machinery, leading to higher hsp synthesis during heat stress (HS) and more rapid resumption of cellular protein synthesis following HS (Dhaubhadel et al., 2002). Genes, other than hsps, associated with varied cellular processes were also up-regulated by EBR in *B. napus* seedlings (Dhaubhadel and Krishna, 2008).

The potential of BRs for increasing plant tolerance against drought, cold and soil salinity had been realized in preliminary studies (Krishna, 2003; Divi and Krishna, 2009). For instance, BR reduced the inhibitory effects of salt on seed germination in *Eucalyptus camaldulensis* (Sasse et al., 1995), rice (Anuradha and Rao, 2001), and *B. napus* (Kagale et al., 2007). In salt sensitive IR-28 rice, EBR reduced the extent of oxidative damage incurred by salt stress, which correlated with less lipid peroxidation, significant increase in the activity of ascorbate peroxidase (APX), higher soluble protein content and higher accumulation of the protective osmolyte proline (Ozdemir et al., 2004). Soaking the roots of *Robinia pseudoacacia* L. seedlings in brassinolide (BL), the most active BR, prior to planting increased the survival and growth of seedlings under simulated drought conditions (Li et al., 2007). These seedlings accumulated higher levels of osmolytes like proline and soluble sugars, had higher leaf water content and greater increases in the activities of antioxidant enzymes as compared to untreated seedlings. A stimulatory effect of BR on the growth of maize and cucumber seedlings was also seen under chilling stress (He et al., 1991; Katsumi, 1991). While these and other similar findings are encouraging, they do not provide the conditions in a model plant system based on which the mechanism of BR-mediated stress tolerance can be investigated in a systematic manner.

We focused on studying the effects of EBR on Arabidopsis, a genetic model system, and *B. napus*, an oil crop plant, under drought, cold and high salt conditions as a means to first confirm this ability of BR and to subsequently use the established experimental conditions to address the mechanism by which BR exerts anti-stress effects. We demonstrated that EBR treatment enhances seedling tolerance to drought and cold stresses in both Arabidopsis and *B. napus*, and helps to overcome a salt stress-induced inhibition of seed germination. The ability of EBR to confer tolerance in plants to a variety of stresses was confirmed through analysis of expression of a subset of drought and cold stress marker genes. Transcriptional changes in these genes were more apparent in EBR-treated Arabidopsis, in particular during earlier time points of stress.

The following chapter describes the standardization of experimental conditions mimicking drought stress, as well as the effects of BR on dehydration stress tolerance in Arabidopsis. EBR-treated Arabidopsis seedlings had greater survival, as compared to

untreated seedlings, under drought stress conditions, as well as higher expression of a subset of drought marker genes.

## 2.2 MATERIALS AND METHODS

### *2.2.1 Plant material and growth conditions*

Arabidopsis ecotype Columbia, seeds were surface-sterilized by sequentially soaking seeds in 75% ethanol for 1 min, rinsing twice in sterile-distilled water, soaking in 1.05% sodium hypochlorite for 20 min with stirring, and rinsing 4-5 times with sterile distilled water. Surface sterilized seed were plated on 1X Murashige and Skoog medium (Sigma, St. Louis) supplemented with B5 vitamins, 1% (w/v) agar, 1% sucrose, and either 1 μM EBR or 0.01% ethanol (solvent for EBR). The plates were kept for 3 days in the dark at 4°C to encourage synchronized germination and then transferred to 22°C with a 16-h photoperiod (80 $\mu E\ m^{-2}\ s^{-1}$) and allowed to grow for 21 days.

### *2.2.2 Stress treatments*

All experiments were repeated three times. For drought stress, 21-day-old Arabidopsis seedlings grown in the presence or absence of EBR were transplanted into pots containing coarse sand. Seedlings were allowed to re-establish growth for 5 days and then subjected to drought stress by withholding water for up to 96 h. Following drought stress, the seedlings were allowed to recover by watering them regularly for the next 2 days. Seedlings that survived and continued to grow were counted. Values in Figure 2.1B represent average percentages of data obtained in three biological replicates. Plant tissue above the sand was collected at various time points during drought stress and quick-frozen.

### *2.2.3 RNA isolation and northern blot analysis*

Total RNA was isolated from frozen plant tissue using TRIzol reagent (Life Technologies, Burlington, ON, Canada). For northern blot analysis, 10 μg of total RNA was separated on a denaturing formaldehyde agarose gel and blotted onto a Biotrans$^{+}$ membrane (ICN Biomedical, Aurora, OH, USA). Blots were hybridized with $^{32}P$-labelled cDNA fragments under the conditions described previously (Krishna et al., 1995).

Following hybridization, the membranes were washed twice in 2 x SSC with 1% SDS at room temperature for 10 min, twice in 0.5 x SSC with 0.5% SDS at 52 $^0$C for 15 min, and if required, twice in 0.1 x SSC with 0.1% SDS at 65 $^0$C for 10 min, and autoradiographed. The cDNAs fragments for *RD29A* (accession no. D13044), *ERD10* (accession no. NM_180616), *RD22* (accession no. D10703), *DEHYDRIN* (accession no. NM_127721), were generated by RT-PCR using RNA isolated from drought-stressed Arabidopsis seedlings. The sequences of these cDNA fragments were verified by sequencing and were $^{32}$P-labelled and used to detect the corresponding transcripts. The blots were stripped and re-hybridized with an 18S ribosomal DNA fragment to indicate RNA loading.

***2.2.4 RT-PCR analysis***

Total RNA (7µg) isolated from unstressed and drought-stressed Arabidopsis was reverse transcribed using the oligo $(dT)_{18}$ primer and SuperScript™ First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). PCR was carried out with an initial denaturation step of 94$^0$C for 4 min followed by various cycles of denaturation (30 s at 94$^0$C), annealing (45 s at 56$^0$C), and extension (1 min at 72$^0$C). After the last cycle, a final extension was carried out for 5 min at 72$^0$C. PCR was performed for 30 cycles for *DREB2A* (accession no. NM_120623), 23 cycles for *ACTIN* (internal control). The following primers were used: *DREB2A-F* 5' TGACGGTACTACTGTGGCTGAG; *DREB2A-R* 5' GTCGCCATTTAGGTCACGTAG; *ACTIN-F* 5' TGCTCTTCCTCATGCTAT; *ACTIN-R* 5' ATCCTCCGATCCAGACACTG.

**2.3 RESULTS**

***2.3.1 EBR increases drought tolerance in Arabidopsis seedlings***

We have found EBR effects on Arabidopsis stress tolerance to be most pronounced when seedlings are grown in the presence of EBR for 21 days (Kagale et al., 2007). Since seedlings receive EBR treatment in petri dishes under sterile conditions, it was not possible to subject them to dehydration stress as such. For this reason,

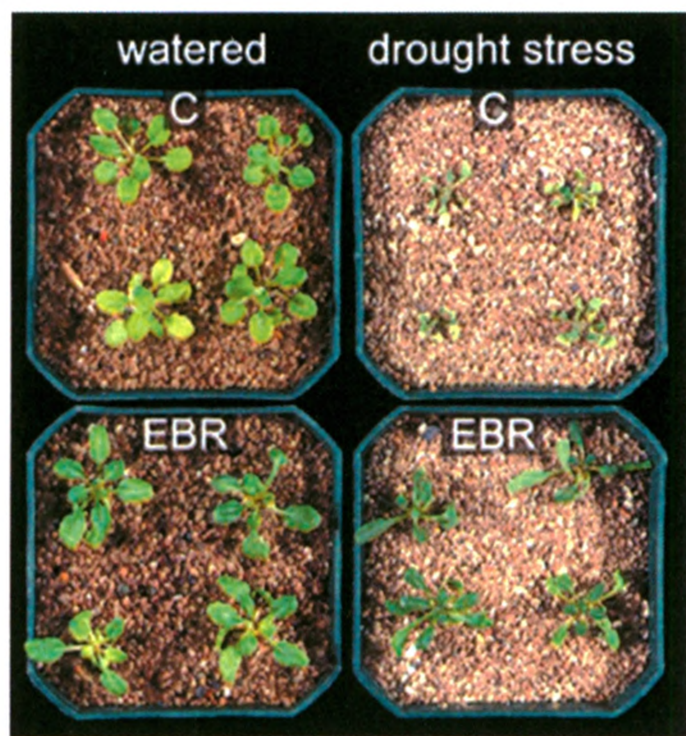
Arabidopsis seedlings grown in the presence or absence of EBR for 21 days were transplanted to sand and then subjected to drought stress as described under materials and methods. The method was standardized for reproducibility and the experiment was repeated three times. Visible morphological changes in response to drought stress, such as leaf wilting, reduction in growth, and complete drying of some seedlings, were frequently observed in untreated seedlings, but were considerably reduced in EBR-treated seedlings (Figure 2.1A). In accordance with this observation, the survival rate of EBR-treated seedlings was noticeably higher than the survival rate of untreated seedlings. About 80% and 28% of EBR-treated seedlings watered at 72 and 80 h after drought stress, respectively, survived and continued to grow. In comparison, only 35% and 7% of the untreated seedlings survived (Figure 2.1B). All of the untreated seedlings were killed by 80-82 h of drought stress, whereas EBR-treated seedlings remained alive past 90 h of drought treatment. These results demonstrate that EBR treatment increases tolerance to drought stress in Arabidopsis seedlings.

### *2.3.2 EBR affects the expression of dehydration-responsive genes*

Response to drought stress is a relatively well-characterized phenomenon in plants, which results in major reprogramming of gene expression (Ramanjulu and Bartels, 2002; Seki et al., 2002). To determine if EBR affects the expression of known dehydration-responsive genes, such as *RD29A*, *ERD10* and *RD22,* transcript levels of these genes were determined in EBR-treated and untreated seedlings under non-stress and dehydration stress conditions. Transcripts of *RD29A* and *ERD10,* encoding late embryogenesis abundant (LEA) proteins (Yamaguchi-Shinozaki and Shinozaki, 1993; Kiyosue, 1994), accumulated to higher levels (2 to 3-fold) in EBR-treated seedlings at earlier time points of stress (up to approximately 60 h of stress), but at later time points the levels were comparable between untreated and treated seedlings (Figure 2.2A). Transcript levels of *RD22,* an abscisic acid (ABA)-dependent dehydration-responsive gene (Iwasaki et al., 1995), switched from being slightly elevated in EBR-treated seedlings at earlier time points to being higher in untreated seedlings at later time point(s). The differences in the expression of these genes at 72 and 84 h, as well as of a

**Figure 2.1** Epibrassinolide (EBR) enhances drought stress tolerance in Arabidopsis. **A)** Arabidopsis seedlings grown in the absence (C) or presence of 1 μM EBR (E) were transplanted into pots containing sand. After 5 days, drought stress was given by withholding water for up to 80 h. Photograph of the plants was taken at 72 h of drought stress. **B)** The graph indicates proportion of plants recovering after being watered at 72 and 80 h of drought stress.

A




B

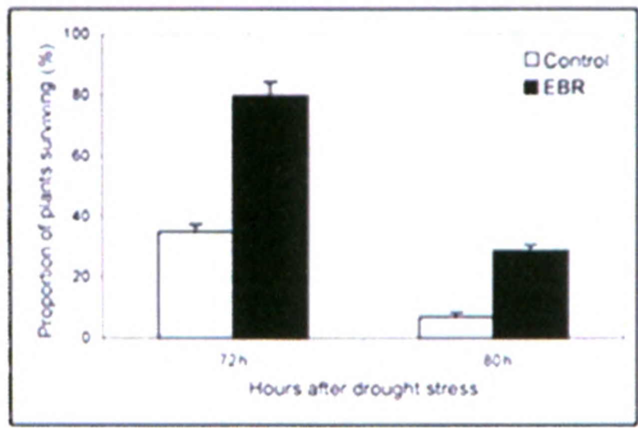
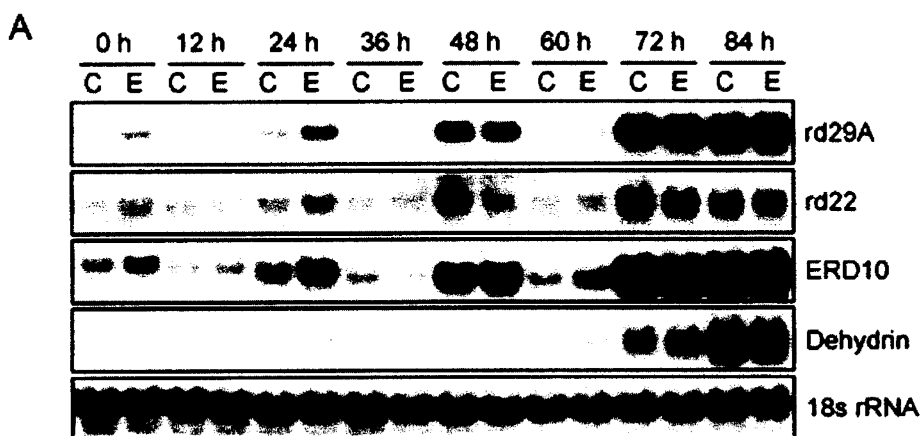
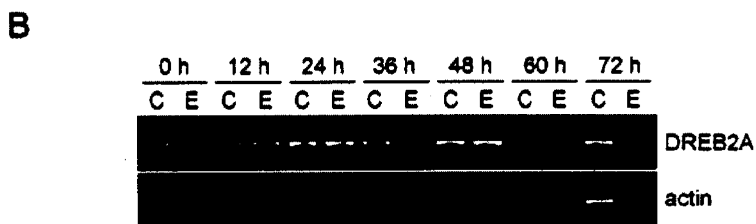


**Figure 2.2** Expression profiles of drought-responsive genes in untreated and EBR-treated Arabidopsis seedlings. **A)** Arabidopsis seedlings were exposed to drought stress (described in materials and methods) for varying lengths of time. Transcript levels of *RD29A*, *RD22*, *ERD10* and a *DEHYDRIN* were analyzed by northern blotting. The expression of 18S rRNA was analyzed to serve as a loading control. **B)** Transcript levels of the transcription factor *DREB2A* was analyzed by RT-PCR. *ACTIN* transcript levels were assayed as controls.

A




B



*DEHYDRIN* gene in the two sets of seedlings did not appear significant. Interestingly, *RD29A*, *ERD10* and *RD22* showed a circadian rhythm expression pattern at early time points of stress when transcript levels were relatively lower. This was overcome by high expression of transcripts at later time points. These experiments were repeated at least two times and the changes described were reproducible.

Transcription factors of the CBF/DREB (cold-responsive element binding factor/drought-responsive element binding factor) family in Arabidopsis bind to *cis*-acting drought-responsive elements (DRE) and regulate gene expression in response to cold, drought and salt stress (Shinozaki and Yamaguchi-Shinozaki, 2000). The mRNA levels of *DREB2A,* a transcription factor involved in drought-responsive gene expression were analyzed by RT-PCR. The expression of *DREB2A* transcripts was comparable between untreated and EBR-treated Arabidopsis seedlings during drought stress conditions (Figure 2.2B).

## 2.4 DISCUSSION

Though BRs have been implicated in drought, cold and salt stress responses (Divi and Krishna, 2009; Krishna, 2003), experimental conditions under which the stress alleviating effects of BR can be studied in a reproducible manner at the morphological level have not been described in literature nor have there been any molecular studies in this direction. Thus, there is a lacuna of convincing evidence of the ability of BR in modulating plant responses to a variety of environmental stresses. Our previous studies have established the role of BR in modulating plant responses to high temperature stress (Dhaubhadel et al., 1999; 2002). The present study established conditions to study the effects of BR on drought stress responses in Arabidopsis in a reproducible manner and also provides the first molecular evidence for a role of BR in drought stress tolerance.

That EBR increased drought tolerance in Arabidopsis seedlings was evident both from the reduction in visible morphological symptoms and enhanced percentage of survival (Figure 2.1A). Similar effects of EBR were seen in *B. napus* seedlings (Kagale et al., 2007). These results confirm the ability of BR to increase drought tolerance in seedlings. Further support to this end comes from the observation that EBR treatment

increased expression of marker genes with known responses to drought and cold stress, as well as of genes believed or demonstrated to enhance drought and cold tolerance of plants. However, unlike ABA, which produces dramatic increases in gene expression, we observed only 2 to 5-fold increases in the expression of some of these genes in response to EBR. This is not surprising since BR-regulated genes on average show expression changes of < 2-fold (Vert et al., 2005). Thus, although BR-induced changes in transcript levels are modest, BR-induced changes in plant phenotypes are clear, implying that either BR controls gene expression at other levels or that the changes affected by BR are together sufficient for inducing the phenotypes.

It was consistently noted that *RD29A*, *ERD10* and *RD22* mRNAs accumulated to higher levels in EBR-treated Arabidopsis seedlings at early time points of drought stress, but were either comparable in the two sets of seedlings or higher in untreated seedlings during later time points. The *RD29A* and *ERD10* genes encode a class of proteins that have molecular chaperone-like functions, preventing protein aggregation during water stress (Goyal et al., 2005). The increase in the transcript levels of these genes at earlier time points suggests that EBR-treated seedlings are likely positioned to tolerate stress better right from the beginning. Due to the positive influence of BR on gene expression at the post-transcriptional level (Dhaubhadel et al., 2002), it is possible that this effect is compounded with further increases at the protein level, at least, for some genes (this could not be tested due to unavailability of antibodies).

Similar to the effect in Arabidopsis, EBR also affected higher expression of dehydration-responsive genes *BNPIP1* and *BnD22* in *B. napus* seedlings (Kagale et al., 2007). *BnPIP1* is an aquaporin gene that is involved in water transportation during germination under stress conditions (Gao et al., 1999). The *BnD22* expression was found to be increased by progressive or rapid water stress that disappeared upon rehydration (Reviron et al., 1992). In another study, Desclos et al. (2008) showed that the water-soluble chlorophyll-binding protein (WSCP) and trypsin inhibitor (TI) activities of BnD22 lead to the protection of younger tissues from adverse conditions by maintaining protein integrity and photosynthesis. In our study, the increase in transcript accumulation of these genes correlated with increased drought tolerance of these seedlings, which, in part, may derive from the proposed functions of these genes, such as better distribution of

water, and efficient defense against the large number of proteases produced during stress conditions, respectively. A possible role for BR in controlling aquaporin activities was previously suggested in an indirect study (Morillon et al., 2001). Though further investigation is required, these results established a correlation between EBR and the expression of one aquaporin gene *BNPIP1* (Kagale et al., 2007).

The expression of structural genes in response to stress must result from a combination of factors - the degree of stress experienced by the seedling depending on its fitness, and the effectiveness by which the seedling can respond to stress. Transcript levels of transcription factors can be taken as an indirect measure of the degree by which the stress response will be launched. Transcript levels of *DREB2A*, a transcription factor that regulates drought-responsive gene expression were determined, but found to be comparable between EBR-treated and untreated Arabidopsis seedlings under no stress and drought stress conditions. This is not surprising given that DREB2 proteins require post-translational activation (Liu et al., 1998). However, in compliance with the higher stress tolerance of EBR-treated *B. napus* seedlings, transcripts of the transcription factors *BNCBF5* and *BNDREB* were present at higher levels in these seedlings, both in the absence of drought stress and under drought conditions (Kagale et al., 2007). Higher expression of transcription factors involved in activating the CBF regulon, which in turn protects plants from drought and cold stresses, supports the idea that EBR-treated seedlings are better primed than untreated seedlings to respond to stress.

In conclusion, the results of the present study along with those of the entire study as published by Kagale et al. (2007), demonstrate that EBR enhances tolerance of seedlings to a variety of abiotic stresses, and that this effect involves changes in the expression of genes encoding both structural and regulatory proteins. Analysis of global gene expression in response to EBR in the future will help in understanding how EBR improves tolerance in plants against a wide range of environmental stresses.

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# CHAPTER 3

## Brassinosteroid-mediated abiotic stress tolerance in Arabidopsis involves interactions with abscisic acid, ethylene and salicylic acid pathways.

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# CHAPTER 3

## 3.1 INTRODUCTION

Brassinosteroids (BRs) are a group of plant steroidal hormones that regulate various aspects of plant growth and development, including cell elongation, photomorphogenesis, xylem differentiation, and seed germination (Sasse, 2003), as well as adaptation to abiotic and biotic environmental stresses (Khripach et al., 2000; Krishna, 2003). Molecular genetic studies of BR-deficient and BR-insensitive mutants have established an essential role for BRs in plant development and led to the identification and characterization of several BR signaling components (Clouse and Sasse, 1998; Gendron and Wang, 2007). A large number of BR-regulated genes have been identified by microarray studies; most of the known BR-regulated genes are associated with plant growth and development, such as cell wall modification, cytoskeleton formation, and hormone synthesis (Vert et al., 2005). How BR regulates gene expression is currently understood for only a small proportion of genes. In the known mechanism of BR-controlled gene expression, BR binding to BRI1 (BRASSINOSTEROID INSENSITIVE1), a plasma membrane localized leucine-rich repeat receptor-like kinase (LRR-RLK), induces association of BRI1 with its co-receptor BAK1 (BRI1-ASSOCIATED KINASE1), which enhances signaling output through reciprocal BRI1 transphosphorylation (Belkhadir et al., 2006; Gendron and Wang, 2007). BRI1 binding to BR inactivates a glycogen synthase kinase-3, BIN2 (BRASSINOSTEROID-INSENSITIVE2), and possibly activates the phosphatase BSU1 (BRI1 SUPPRESSOR1). BIN2 negatively regulates transcription factors BZR1 (BRASSINAZOLE RESISTANT1) and BES1 (BRI1 EMS SUPPRESSOR) by phosphorylating them, while BSU1 positively regulates BR signaling by dephosphorylating BZR1 and BES1. Activated BZR1 and BES1 accumulate in the nucleus and directly bind to CGTG(T/C)G motif in the promoters of BR biosysnthesis genes *CPD* and *DWF4* (He et al., 2005) and to the E boxes (CANNTG) of the *SAUR-ACI* promoter, respectively, to affect gene expression (Yin et al., 2005). The recent demonstrations that BES1 interacts with other transcription factors such as BIMs (BES1-INTERACTING MYC-LIKE) (Yin et al., 2005), MYB30 (MYB DOMAIN PROTEIN 30), which acts as a positive regulator of the hypersensitive cell-death response (Vailleau et al., 2002), and the jumonji (Jmj) domain-

containing proteins ELF6 (EARLY FLOWERING 6) and REF6 (RELATIVE OF EARLY FLOWERING 6) that are involved in regulating flowering time (Yu et al., 2008), points to recruitment of different proteins by BES1 as one of the ways by which BR affects diverse biological processes.

The role of BRs in plant stress responses has been confirmed in several studies (Dhaubhadel et al., 1999; 2002; Kagale et al., 2007; Koh et al., 2007). BR promotes tolerance in plants to a wide range of stresses, including heat, cold, drought and salinity, and this increase is generally correlated with higher expression of stress marker genes, such as *heat shock protein* (*hsp*) genes, *RESPONSIVE TO DESSICATION29A (RD29A)*, and *EARLY RESPONSIVE TO DEHYDRATION 10* (*ERD10)* (Dhaubhadel et al., 1999; Kagale et al., 2007), indicating that increased expression of stress-responsive genes is responsible, in part, for the higher stress tolerance in BR-treated plants. The mechanisms by which BR controls plant stress responses and regulates the expression of stress response genes are not known. Since different plant hormones can regulate similar physiological processes, and cross-talk between different hormones can occur at the level of hormone biosynthesis, signal transduction or gene expression (Nemhauser et al., 2006), it was proposed that BR regulates plant stress responses via cross-talk with other hormones (Krishna, 2003).

The plant growth regulators with documented roles in plant adaptation to abiotic and biotic stresses are abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and salicylic acid (SA). SA, JA and ET are important in defense against pathogen and pest attack (Bari and Jones, 2009), whereas ABA is a key molecule involved in salt and drought stress (Zhu, 2002). SA, ET, ABA and JA have also been linked to heat stress. Studies of hormone deficient and insensitive mutants have demonstrated the involvement of SA, ET and ABA in acquired thermotolerance of plants (Larkindale et al., 2005), and additionally for SA and JA, a role in basal thermotolerance of plants (Clarke et al., 2004; 2009). Although experimental evidence points to interactions of BR with auxin (Mouchel et al., 2006; Hardtke et al., 2007), gibberellic acid (GA) (Bouquin et al., 2001; Shimada et al., 2006), ABA (Steber and McCourt, 2001; Abraham et al., 2003), ET (Yi et al., 1999; Arteca and Arteca, 2001) and JA (Kitanaga et al., 2006; Müssig et al., 2006), the relationship of BR with these hormones has been documented primarily in plant growth

regulatory processes. Furthermore, with the exception of BR-auxin interaction, little is known in terms of genes how BR interacts with other hormones. Recent progress made towards understanding BR-auxin interaction can serve as a paradigm for how two hormones could interact at multi-levels. Auxin and BR share a number of target genes, many of which are involved in growth-related processes (Hardtke et al., 2007). Since promoter regions in BR-responsive genes are enriched in Auxin Response Factor (ARF)-binding sites, and binding sites of BES1 are over-represented in genes regulated by both hormones, regulatory elements in gene promoters represent a point of cross-talk between auxin and BR (Nemhauser et al., 2004). Recently, the BR-regulated BIN2 kinase was demonstrated to phosphorylate ARF2, a member of the ARF family of transcriptional regulators, leading to loss of ARF2 DNA binding and repression activities (Vert et al., 2008). Thus, in this model ARF2 links BR and auxin signaling pathways. In addition to gene coregulation, BR can also promote auxin transport (Li et al., 2005), and optimal auxin action is dependent on BR levels (Mouchel et al., 2006).

The role of BR in plant responses to abiotic stress has become well established over the last decade, but there are very few reports indicating how BR interacts with other stress-related hormones and their signaling pathways in conferring stress tolerance. While there exists evidence to indicate that BR increases ET and JA levels under normal growth conditions (Kitanaga et al., 2006; Arteca and Arteca, 2001), there appears to be only one report linking BR with increase in ABA levels in the lower plant *Chlorella vulgaris* under stress condition (Bajguz, 2009). Very recently it has been demonstrated that ABA inhibits BR signaling through phosphorylation of BES1 (Zhang et al., 2009). Currently there are no studies at the genetic level as to how BR interacts with other hormones under stress conditions. Here we asked the question whether one or more stress-related hormones, such as ABA, ET, JA or SA, have a major role in BR-mediated stress tolerance. Arabidopsis mutants with either disrupted or enhanced hormone pathways were tested for phenotypes and gene expression in response to BR under high temperature and high salt conditions. Our results indicate that in Arabidopsis the NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) is a critical component of BR-mediated effects on thermotolerance and salt tolerance, that BR exerts anti-stress effects both

independently as well as through interactions with other hormones, ABA inhibits BR effects during heat stress, and that BR shares transcriptional targets with other hormones.

## 3.2 MATERIALS AND METHODS

### *3.2.1 Plant material and growth conditions*

The *eds5-1* (CS3735), *npr1-1* (CS3726), *cpr5-2* (CS3770), *ein2* (CS8844), *eto1-1* (CS3072), *aos* (CS6149), and *jar1-1* mutants belong to the Columbia (Col) background, while the *aba1-1* (CS21) and *abi1-1* (CS22) mutants are in the Landsberg *erecta* (L*er*) background. Accordingly, the WT Col and L*er* were used as controls in the experiments. The *aos* mutant is a T-DNA knockout line derived from Col-6, hence its parental line (CS8155) was used as control. The *jar1-1* mutant was a kind donation from Dr. Pradeep Kachroo (University of Kentucky, Lexington, KY, USA). All other mutants were obtained from the Arabidopsis Biological Resource Center (ABRC).

Seedlings were grown essentially as described by Kagale et al. (2007). Seeds were surface sterilized and plated on 1X Murashige and Skoog medium (Sigma, St. Louis) supplemented with B5 vitamins, 1% (w/v) agar, 1% sucrose, and either 1 μM EBR or 0.01% ethanol (solvent for EBR). The plates were kept for 3 days in the dark at $4^0$C to encourage synchronized germination and then transferred to $22^0$C with a 16/8 h photoperiod (80 μE $m^{-2}$ $s^{-1}$) and allowed to grow for 21 days. For the short-term EBR treatment, 21-day-old seedlings grown in the medium without EBR were submerged for 7 h in sterile water containing 1 μM EBR or 0.01% ethanol.

### *3.2.2 Heat stress treatments*

The thermotolerance of Arabidopsis seedlings was assayed according to Kagale et al. (2007) with minor modifications. Seedlings grown at $22^0$C for 21 days were exposed to $43^0$C for 4 h and scored as dead or alive after 7 days of recovery at $22^0$C. Values in Figure 3.1B represent average percentages of data obtained in three biological replicates. Plant tissue was collected at different time points and quick-frozen for protein isolation and for the TBARS assay.

### *3.2.3. Salt stress treatments*

Surface sterilized seeds were germinated on 0.5X Murashige and Skoog medium supplemented with B5 vitamins and either 1 μM EBR or 0.01% ethanol. Salt treatment was given by including 150 mM NaCl in the medium. All plates were kept for 3 days in the dark at $4^0$C plates and then transferred to $22^0$C. Percent germination was determined 3 days after transferring plates to $22^0$C. Seeds with emerging cotyledons were scored as germinated. Percent survival was calculated by counting the number of seedlings that showed true leaves and green colour at 20 days after imbibition. Values in Figure 3.5 are average percentages of data obtained in three biological replicates.

### *3.2.4 Thiobarbituric Acid Reactive Substance (TBARS) assay*

TBARS assay was performed according to Heath and Packer (1968). Seedlings grown at $22^0$C for 21 days were subjected to $43^0$C for 3 h and then allowed to recover for 2 days at $22^0$C. Seedlings were quick frozen in liquid nitrogen and 0.5 g of the tissue was ground in 1 mL of solution containing buffer 1 and buffer 2 in equal proportions (buffer 1: 0.5 mL of 0.5% [w/v] thibarbituric acid in 20% [v/v] trichloroacetic acid; buffer 2: 0.5 mL 175 mM Nacl in 50 mM Tris, pH 8.0). Ground samples were heated to $94^0$C for 1 h, centrifuged at 13,000 rpm for 20 min, and the absorbance of the supernatant was measured at 532 and 600 nm. The levels of TBARS were deduced from the malonaldehyde standard curve and in each case the TBARS levels in the EBR-treated samples were compared to that of untreated control. Values in Figure 3.2A were averaged from three replicates.

### *3.2.5 Protein extraction and western blotting*

Extraction of total proteins and western blotting were carried out as described by Dhaubhadel et al. (1999). Seedlings grown at $22^0$C for 21 days were either maintained at $22^0$C or subjected to $43^0$C for 3 and 4 h. Seedling tissue above the medium was harvested, frozen in liquid nitrogen and stored at $-80^0$C. Frozen tissue was ground in protein extraction buffer [25 mM Tris-HCL, 1 mM EDTA, 20 mM NaCl, 1 mM PMSF, 1 mM benzamidine, 1 μg/ml leupeptin and 2 μg/ml aprotinin] and after centrifugation at 13,000 rpm for 30 min, the supernatant was transferred to a new tube. Protein

concentration was determined by the bradford assay. Total proteins (15 μg) were separated on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane by electroblotting using the Trans-blot Semi-Dry Electrophoretic Transfer Cell (BioRad, Hercules, CA, USA). Hsp90 was detected by sequential incubation with the polyclonal R2 antisera (Krishna et al., 1997) and the peroxidase conjugated anti-rabbit IgG, each at a dilution of 1:5,000, followed by chemiluminescent detection (ECL system, Amersham, Baie d'Urfe, QC).

### *3.2.6 RT-PCR analysis*

RNA was extracted from 21-day-old seedlings grown at $22^0$C using the SV Total RNA Isolation System (Promega, Madison, WI). Total RNA (3 μg) was reverse transcribed using the oligo $(dT)_{18}$ primer and Super Script First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR was carried out with an initial denaturation step of $94^0$C for 5 min followed by various cycles of denaturation (40 s at $94^0$C), annealing (45 s at $53^0$C), and extension (45 s at $72^0$C). After the last cycle, a final extension was carried out for 5 min at $72^0$C. PCR was performed for 32 cycles for *PDF1.2, HEL, WRKY70, LTP4, RD22;* 35 cycles for *LOX2, PR-1, WAK1, GST1*; and 21 cycles for *ACTIN* (control gene).

The following primers were used:

***PR-1-F***: 5' GATGTGCCAAAGTGAGGTG,

***PR-1-R***: 5' CTGATACATATACACGTCC,

***WRKY70-F***: 5' CGCCGCCGTTGAGGGATCTC,

***WRKY70-R***: 5' CGCCGCCACCTCCAAACAC,

***WAK1-F***: 5' GAGTTACTTTGCGACTGCCA,

***WAK1-R***: 5' CAGCTTCCTGGATCTCCTTC,

***PDF1.2-F***: 5' AATGAGCTCTCATGGCTAAGTTTGCTT,

***PDF1.2-R***: 5' AATCCATGGAATACACACACGATTTAGCACC,

***LOX2-F***: 5' CTCTTCAGAGCACGCTACG,

***LOX2-R***: 5' GAAGATGGAGGGAAGAGCTG,

***HEL-F***: 5' ACAAGGCCATCTCATTGTTG,

*HEL-R*: 5' GATCAATGGCCGAAACAAG,

*GST1-F*: 5' TTGGCTTCTGACCACTTCAC,

*GST1-R*: 5' ACGCTCGTCGAAGAGTTTCT,

*RD22-F*: 5' GCGAGCTAAAGCAGTTGCGGTATG,

*RD22-R*: 5' CGGCTAGTAGCTGAACCACACAAC,

*LTP4-F*: 5' CACCAACTGCGCCACCATCAAG,

*LTP4-R*: 5' GCCATCAAGACAAACAAAGAC,

*DWF4-F*: 5' ACGGAGCAAATTCTCGATC,

*DWF4-R*: 5' AGCTCTTCAACGGCTTTAG,

*ACTIN-F*: 5' TGCTCTTCCTCATGCTAT,

*ACTIN-R*: 5' ATCCTCCGATCCAGACACTG,

## 3.3 RESULTS

### *3.3.1 EBR effects on basal thermotolerance in different hormone genotypes*

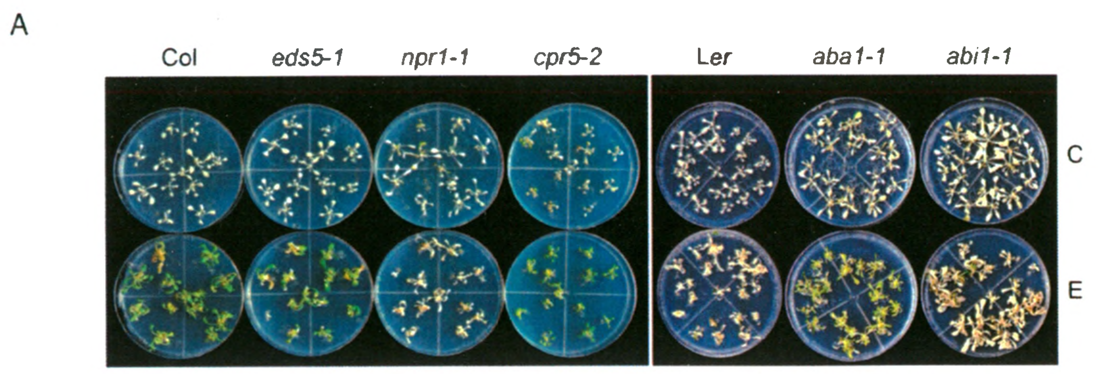
We have previously demonstrated that EBR enhances the basic thermotolerance of *Brassica napus*, tomato (Dhaubhadel et al., 1999; 2002) and Arabidopsis seedlings (Kagale et al., 2007). Since the effects of EBR on stress tolerance are most pronounced when seedlings are grown in the presence of EBR for 21 days (long-term treatment), we postulated the involvement of other phytohormones in this process (Krishna, 2003). Several hormone pathways, such as of ABA, ET, SA and JA, have been linked with one or more environmental stresses, including heat stress (HS). We therefore evaluated the effects of EBR on thermotolerance in a subset of Arabidopsis hormone mutants altered in either biosynthesis or signaling of these phytohormones (Table 3.1). We first studied the effects of EBR on basal thermotolerance in SA genotypes *npr1-1* (defective in SA signaling), *eds5-1* (defective in SA synthesis), and *cpr5-2* (SA-overproducer) by exposing 21 day old seedlings to $43^0$C for 4 h, allowing them to recover at $22^0$C for 7 days and then scoring for dead and alive seedlings. WT, *eds5-1*, *npr1-1* and *cpr5-2* seedlings grown in the absence of EBR had average survival rates of 7.5%, 4.5%, 5.5% and 13.5%, respectively, and those grown in the presence of EBR had survival rates of 69.3%, 58.3%, 13.4% and 83%, respectively (Figure 3.1A and B). From these results it is clear that the survival rates of WT and different SA genotypes were significantly

**Table 3.1** Mutant alleles used in the study with the description of corresponding genes and mutant phenotypes.

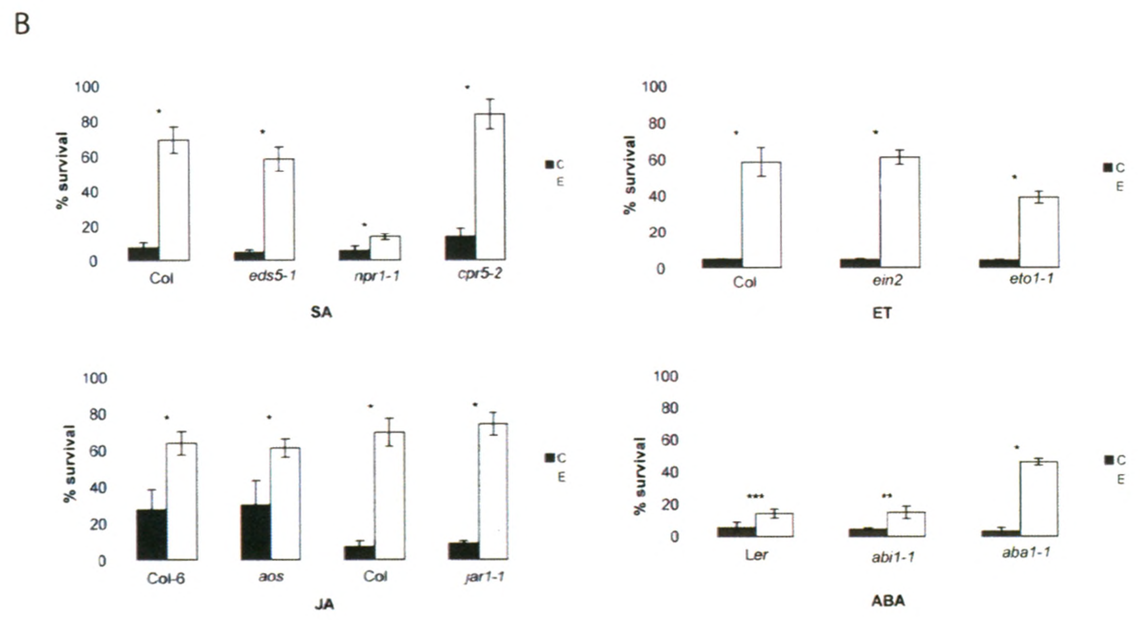
| Category | Locus (AGI) | Gene | Description | Mutant allele |
|---|---|---|---|---|
| SA synthesis defective | At4g39030 | *EDS5* | member of the MATE-transporter family; mutants do not accumulate SA after pathogen inoculation and are hypersusceptible to pathogen infection (Glazebrook et al., 1996; Rogers et al., 1997; Nawrath et al., 1999) | *eds5-1* |
| SA-insensitive | At1g64280 | *NPR1* | similar to the transcription factor inhibitor I kappa B, and contains ankyrin repeats; key regulator of SA-mediated systemic acquiredresistance (SAR) pathway; mutants are SA-insensitive and hypersusceptible to pathogen infection (Cao et al., 1994; Delaney et al., 1995) | *npr1-1* |
| High SA levels | At5g64930 | *CPR5* | regulator of expression of pathogenesis-related (*PR*) genes; participates in signal transduction pathways involved in plar defense; mutants exhibit increased SA levels and constitutive expression of *PR* genes (Bowling et al., 1997; Boch et al., 1998 ) | *cpr5-2* |
| ET-insensitive | At5g03280 | *EIN2* | NRAMP metal transporter family; involved in ET signal transduction;mutants are ET-insensitive (Guzman and Ecker, 1990) | *ein2* |
| High ET levels | At3g51770 | *ETO1* | encodes a negative regulator of 1-aminocyclopropane-1-carboxylic acid synthase5(ACS5), which catalyzes the rate-limiting step in ET biosynthesis; mutations elevate ET biosynthesis by affecting the posttranscriptional regulation of ACS (Guzman and Ecker, 1990) | *eto1-1* |
| JA-deficient | At5g42650 | *AOS* | encodes a member of the cytochrome p450 CYP74 gene family that functions as an allene oxide synthase; catalyzes dehydration of the hydroperoxide to an unstable allene oxide in the JA biosynthetic pathway; mutants are JA-deficient (Park et al., 2002) | *aos* |
| JA-insensitive | At2g46370 | *JAR1* | encodes cytoplasmic localized phytochrome A signaling component protein similar to the GH3 family of proteins; loss of function mutants are defective in a variety of responses to JA (Staswick et al., 1992) | *jar1-1* |
| ABA-deficient | At5g67030 | *ABA1* | encodes zeaxanthin epoxidase gene that functions in first step of ABA biosynthesis; mutants are ABA-deficient (Koornneef et al., 1982) | *aba1-1* |
| ABA-insensitiv | At4g26080 | *ABI1* | Protein phosphatase 2C; involved in ABA signal transduction; mutants are ABA-insensitive (Koornneef et al., 1984) | *abi1-1* |

**Figure 3.1** Effect of EBR treatment on basal thermotolerance of Arabidopsis wild-type (WT) and SA, ET, JA and ABA mutant seedlings. **A)** WT and mutant seedlings grown on a nutrient medium in the absence (C) or presence of 1 μM EBR (E) were exposed to $43^0$C for 4 h. Photographs of the seedlings were taken after recovery at $22^0$C for 7 days. **B)** Proportion of WT and mutant seedlings surviving the lethal heat stress ($43^0$C for 4 h). Seedlings grown and treated as described in **(A)** were scored as dead or surviving after 7 days of recovery at $22^0$C. WT controls were L*er* for *abi1-1* and *aba1-1*, Col-6 for *aos*, and Col for all other mutants. Data shown are average of three replicates. Error bars represent standard error (SE) of mean for three replicates. The threshold of significance as analyzed by student's t-test is indicated above the histograms (**p* <0.01; ***p* <0.05; ****p* =0.07).

A




B



increased by EBR treatment, but the increase was considerably less in case of *npr1-1* as compared to other genotypes within this group

For genotypes related to ethylene, EBR increased survival rates of WT, *ein2* (ET-insensitive), and *eto1-1* (ET-overproducer) seedlings to significant levels as compared to seedlings with no treatment (Figure 3.1B). The JA mutants *aos* (JA-deficient) and *jar1-1* (defective in JA response) belong to different backgrounds; *jar1-1* is in Arabidopsis ecotype Col, whereas *aos* is in Col-6 background. Under the HS conditions employed here, untreated WT Col-6 survived better than untreated WT Col. EBR effect on seedling survival was more pronounced in Col than in Col-6 background (Figure 3.1B). EBR increased survival rates of *aos* and *jar1-1* seedlings to amounts similar to corresponding WT seedlings (Figure 3.1B).

The ABA mutants *aba1-1* (ABA-deficient) and *abi1-1* (ABA-insensitive) are from the L*er* background; hence, WT L*er* was used for comparison with these mutants. Under the conditions used, EBR was less effective in WT L*er* as compared to WT Col (about 2.5-fold increase in L*er* *vs.* 9-fold increase in Col in survival rates in response to EBR) (Figure 1B). Since ABA has been linked with heat tolerance (Larkindale and Knight, 2002; Larkindale et al., 2005), we expected ABA mutants to be less thermotolerant than WT even in the presence of EBR. Contrary to our expectation we found that the effect of EBR was most distinct in *aba1-1* (survival rate of 43.3%) as compared to WT (14.3%) and *abi1-1* (15%) seedlings (Figure 3.1A and B). These results suggest that ABA masks BR effects on the HS response pathway of WT Arabidopsis seedlings.

From the survival data represented in Figure 3.1 it is clear that when EBR effect in any hormone genotype is viewed in reference to the effect on the corresponding WT, EBR could increase the basal thermotolerance of all hormone genotypes, but its effect was minimal in *npr1-1*. Since the SA-deficient *NahG* transgenic line (Gaffney et al., 1993) and the JA response defective *coi1* mutant (Feys et al., 1994) could not be obtained for this study, we cannot yet conclude that SA and JA are dispensable for BR-mediated increase in thermotolerance. However, from the collection of mutants used here it would appear that BR can exert anti-stress effects that are independent of ABA, ET, JA and SA, at least to some extent. The dependency of BR on NPR1 in mediating stress tolerance is a

first time observation made in this study. Whether BR modulates NPR1 activity via SA or BR pathway or both remains to be determined.

### *3.3.2 EBR effects on oxidative damage in hormone mutants*

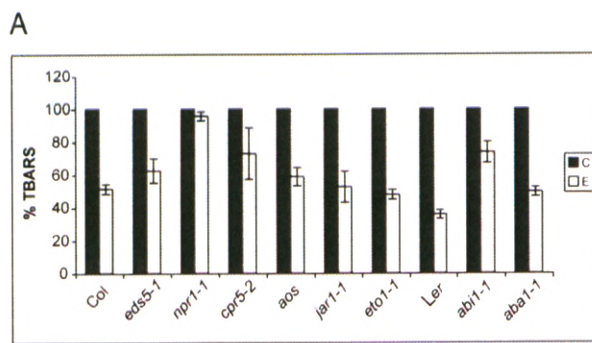
Heat stress produces oxidative damage, which as a result of lipid peroxidation leads to the production of thiobarbituric acid reactive substances (TBARS) (Heath and Packer, 1968; Larkindale et al., 2005). To complement the results of the HS phenotype of seedlings, oxidative damage levels were assessed in untreated and EBR-treated seedlings. Measurements of TBARS in time course experiments determined that maximum oxidative damage occurred post HS (during the recovery period). Therefore, seedlings exposed to HS and then allowed to recover for 2 days at $22^0C$ were used for TBARS analysis. With the exception of *npr1-1,* EBR treatment reduced the levels of oxidative damage in WT and other mutant seedlings as compared to their untreated counterparts (Figure 3.2A). WT Col and L*er* exhibited 45% and 60% reduction in TBARS production, respectively, while the mutant seedlings showed 25-50% less TBARS in response to EBR treatment. It should be noted that the data shown in Figure 3.2A do not allow a comparison of oxidative damage across genotypes, but rather a comparison of oxidative damage levels between untreated and treated seedlings of the same genotype. Consistent with its lower survival rate (Figure 3.1B), the *npr1-1* mutant showed an insignificant 4.5% reduction in TBARS production in response to EBR treatment (Figure 3.2A). However, in contrast to the relatively higher survival of *cpr5-2* seedlings, the reduction in oxidative damage in these seedlings in response to EBR measured only 25% relative to no treatment. Overall, these results demonstrated that EBR treatment can reduce oxidative damage during HS and that this effect is not critically dependent on any one hormone in question, although a functional NPR1 protein appears to be required for a complete effect of EBR on thermotolerance of seedlings.

### *3.3.3 EBR induces higher accumulation of hsp90 in aba1-1*

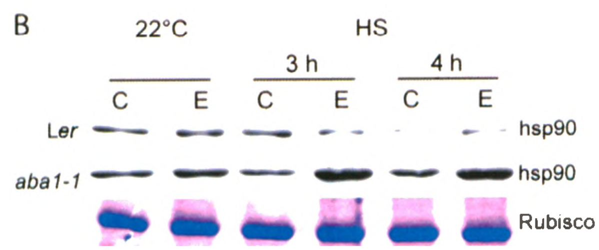
We have found that EBR treatment leads to significant increases in the levels of hsps during HS in *B. napus* (Dhaubhadel et al., 1999; 2002), but the effect of EBR on hsp levels in Arabidopsis is subtle (Kagale et al., 2007). We wished to see how EBR would

**Figure 3.2** Effect of EBR treatment on heat induced-oxidative damage and hsp90 accumulation in WT and mutant seedlings. **A)** Percent TBARS was measured for untreated (C) and EBR-treated (E) WT and mutant seedlings exposed to $43^0$C for 3 h and allowed to recover at $22^0$C for 2 days. Data shown are average of three replicates. Error bars represent SE of mean for three replicates. The differences between the (C) and (E) values for *npr1-1* and *cpr5-2* were not significant, but for all other genotypes the differences were significant to $p$ <0.02. **B)** Total protein was isolated from WT (L*er*) and *aba1-1* seedlings grown in the absence (C) or presence (E) of EBR at $22^0$C and either maintained at $22^0$C or exposed to $43^0$C for 3 h and 4 h (HS). Samples were analyzed by Western blotting using an anti-hsp90 antibody. Coomassie blue staining of ribulose-1, 5-biphosphate carboxylase/oxygenease (rubisco) was used as loading control.

A




B



affect the accumulation of hsp90 in various mutant seedlings in the absence of HS ($22^0$C) and in response to HS (3 h and 4 h exposure to $43^0$C). With the exception of *aba1-1* (Figure 3.2B), no significant differences in the steady state levels of hsp90 were observed between EBR-treated and untreated mutant seedlings, including *npr1-1*. EBR-treated *aba1-1* seedlings accumulated approximately 3- and 2.5-fold higher levels of hsp90 at 3 and 4 h of HS, respectively, as compared to untreated *aba1-1* seedlings (Figure 3.2B). By contrast, EBR-treated WT seedlings showed a maximum of 1.3-fold increase in hsp90 levels at 4 h of HS as compared to untreated seedlings. The fold change values are average of three different experiments, which consistently produced the same pattern. Thus, with respect to higher survival following HS and greater accumulation of hsp90 during HS, EBR produced most distinct effects in the ABA-deficient *aba1-1* mutant. These results reinforce the idea that ABA suppresses BR effects in WT seedlings. Although convincing evidence for antagonism between BR and ABA in plant growth regulatory processes, such as germination has been provided before (Steber and McCourt, 2001; Zhang et al., 2009), the demonstration at the genetic and molecular levels of an antagonistic relationship between the two hormones in plant stress response is new.

### *3.3.4 EBR up-regulates the expression of SA, JA/ET and ABA response genes in both WT and corresponding mutants*

Plant hormone responses in Arabidopsis have been correlated with the expression of hormone–specific marker genes. We studied the expression of few such genes that are well documented to be induced by SA, JA/ET or ABA, both before and after treatment with EBR. The *PATHOGENESIS-RELATED1* (*PR-1*), the transcription factor *WRKY70*, and the *WALL-ASSOCIATED KINASE1* (*WAK1*) genes are known to be regulated primarily by SA (Uknes et al., 1992; He et al., 1998; Li et al., 2004); *PLANT DEFENSIN1.2* (*PDF1.2*), *LIPOOXYGENASE2* (*LOX2*) and *HEAVIN-LIKE PROTEIN* (*HEL*) by JA/ET (Potter et al., 1993; Bell et al., 1995; Penninckx et al., 1996); and *RESPONSIVE TO DESSICATION22* (*RD22*) and *LIPID TRANSFER PROTEIN4* (*LTP4*) by ABA (Iwasaki et al., 1995; Arondel et al., 2000).

The steady-state levels of *PR-1*, *WRKY70* and *WAK1* transcripts were elevated by EBR in WT and SA-related genotypes, including *npr1-1*, albeit at different levels (Figure 3.3A). The *npr1-1* genotype has been noted previously to be defective in the expression of *PR* genes (Cao et al., 1994). Full-scale induction of *WRKY70* and *WAK1* by SA also requires a functional NPR1 (He et al., 1998; Li et al., 2004). Our results clearly indicate that EBR can mediate induction of *PR-1*, *WRKY70* and *WAK1* to levels seen in Figure 3.3A in an NPR1-independent manner.

EBR treatment also enhanced the expression of the JA/ET marker gene *PDF1.2* in WT, *aos*, *jar1-1* and *eto1-1* backgrounds, but not to the same extent in *ein2* (Figure 3.3A). The effect of EBR on *LOX2* expression was distinct in Col and *jar1-1* backgrounds, but not in Col-6 and *aos* backgrounds. Increase in the expression of the *HEL* gene by EBR was only slight.

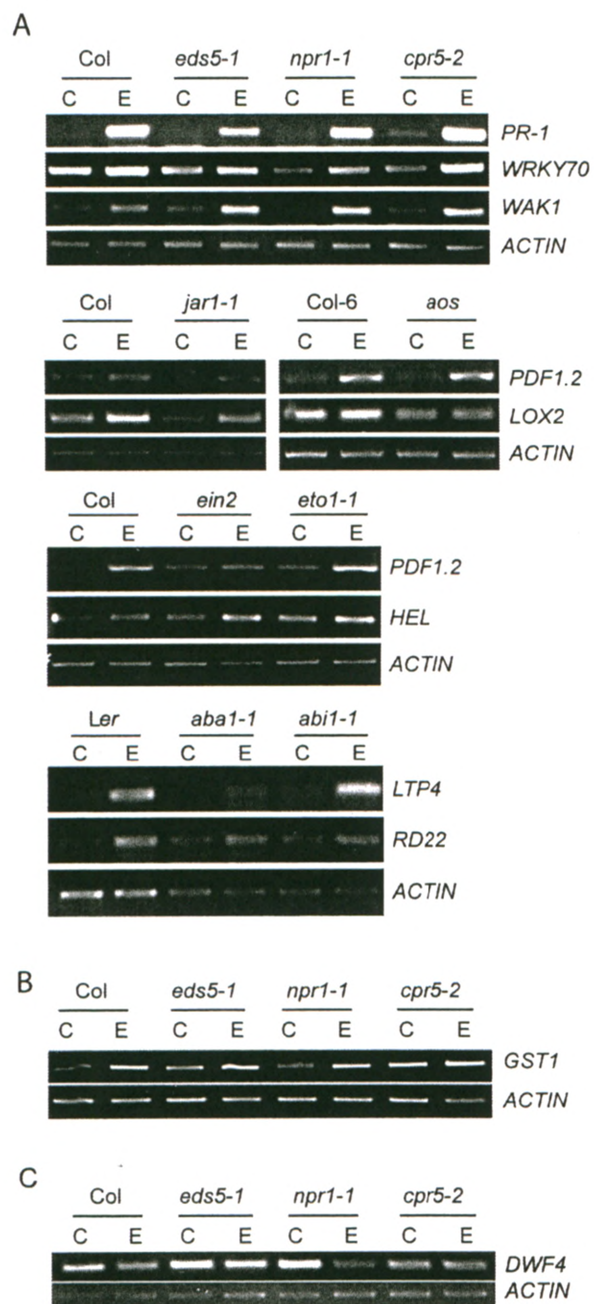
The ABA-responsive *LTP4* showed dramatic induction by EBR in WT and *abi1-1* background (Figure 3.3A), but not in *aba1-1*. The transcript levels of the ABA-marker gene *RD22* were up-regulated by EBR only slightly in *aba1-1* and *abi1-1* mutant seedlings, but significantly in WT (Figure 3A), indicating interaction between ABA and BR in affecting gene expression.

To determine the interaction of EBR with SA, JA/ET and ABA in the regulation of *GST1*, a gene common to abiotic stress and defense pathways (Wagner et al., 2002), we compared its transcript levels in untreated and EBR-treated WT and mutant seedlings. EBR enhanced *GST1* transcript levels in SA (Figure 3.3B), as well as in all other hormone genotypes studied (Appendix 3.1). As would be expected, *cpr5-2* had the highest expression of *GST1* even in the absence of EBR.

Exogenous BR negatively regulates the BR biosynthetic gene *DWF4* (Tanaka et al., 2005). To ensure that transport, perception and signaling of BR is intact in *npr1-1*, which was most inert to EBR effects, *DWF4* expression was determined in WT and *npr1-1*. The fact that *DWF4* levels were reduced in EBR-treated WT and *npr1-1* seedlings as compared to untreated seedlings (Figure 3.3C) suggests that the BR pathway is intact in *npr1-1*.

Taken together, these results demonstrate that most, if not all, of the SA, JA/ET and ABA -responsive genes tested in the present study are also up-regulated by BR both

**Figure 3.3** Effect of EBR on the expression of SA, JA/ET and ABA response genes. Total RNA was isolated from WT and mutant seedlings grown in the absence (C) or presence (E) of EBR at $22^0$C, and transcript levels were analyzed by RT-PCR. Actin was included as a control for constitutive expression. **A)** Expression profiles of *PR-1, WRKY70,* and *WAK1* in WT and SA-related genotypes; *PDF1.2* and *LOX2* in WT and JA mutants; *PDF1.2* and *HEL* in ET-related genotypes; *LTP4* and *RD22* in ABA mutants. **B)** Expression profile of the stress-responsive *GST1* in WT (Col) and SA genotypes. **C)** Down-regulation by EBR of *DWF4* in WT (Col) and SA-insensitive *npr1-1* mutant.


A
Col
eds5-1
npr1-1
cpr5-2
C E C E C E C E
PR-1
WRKY70
WAK1
ACTIN
Col
jar1-1
Col-6
aos
C E C E C E C E
PDF1.2
LOX2
ACTIN
Col
ein2
eto1-1
C E C E C E
PDF1.2
HEL
ACTIN
Ler
aba1-1
abi1-1
C E C E C E
LTP4
RD22
ACTIN
B
Col
eds5-1
npr1-1
cpr5-2
C E C E C E C E
GST1
ACTIN
C
Col
eds5-1
npr1-1
cpr5-2
C E C E C E C E
DWF4
ACTIN

in WT and mutant backgrounds, albeit to different levels. These results point to overlapping gene targets of ABA, JA/ET or SA and BR, as well as to hormone interactions controlling the final output.

### *3.3.5 Up-regulation of a subset of genes by EBR is a direct effect of EBR*

Since the effect of EBR on thermotolerance is best seen when plants are grown in the presence of EBR for 2-3 weeks, our gene expression studies were also conducted in plants receiving a long-term exposure to EBR. To determine whether the effect of EBR on SA, JA/ET and ABA -responsive genes is direct, we studied the expression of a subset of genes in response to a short-term treatment with EBR. It is to be noted that up-regulation of genes by BR can take as long as 18-48 hrs even in short-term treatment (Zurek et al., 1994; Dhaubhadel and Krishna, 2008). Similar to the results of long-term treatment, the 7 h short-term treatment with EBR significantly enhanced the transcript levels of *PR-1* and to a lower extent the levels of *RD22* and *GST1* (Figure 3.4), suggesting a direct role of EBR in modulating the transcript levels of these genes. Although a small increase in the expression of *PDF1.2* was seen in replicate experiments, additional time points will be required to conclusively determine EBR effects on *PDF1.2* expression in short-term treatment. Down-regulation of *DWF4* by exogenous BR was used as an experimental control (Figure 3.4).

### *3.3.6 EBR rescues hypersensitivity of ein2 to inhibition of germination by salt stress*

We have previously shown that EBR helps to overcome salt stress-induced inhibition of seed germination in *B. napus* (Kagale et al., 2007). More recently it was demonstrated that BR-deficient and BR signaling-defective mutants are more inhibited in germination on salt while a BRI1 overexpressing transgenic line is more resistant to salt than WT (Zhang et al., 2009). To study the effects of EBR on salt stress in hormone mutants, we selected *ein2* because of its hypersensitivity to salt stress (Wang et al., 2007), and *eto1-1* and *npr1-1* because of their relatively higher susceptibility to HS even after EBR treatment. Seeds were allowed to germinate on 150 mM NaCl in the presence or absence of EBR, and seedlings with emerged cotyledons were scored after 3 days. Inhibition of germination of *ein2* seeds by salt was significantly reduced in the presence

**Figure 3.4** Effect of EBR on the expression of SA, JA/ET and ABA response genes after short-term treatment with EBR. WT (Col) seedlings grown for 21 days in the absence of EBR were treated with 1 μM EBR (E) or 0.01% ethanol (C) for 7 h. Transcript levels of *PR-1*, *PDF1.2*, *RD22*, *GST1*, *DWF4* and *ACTIN* were analyzed by RT-PCR. *DWF4* (down-regulation) was used as a control for BR-regulated gene expression.

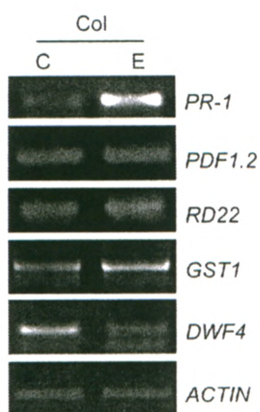
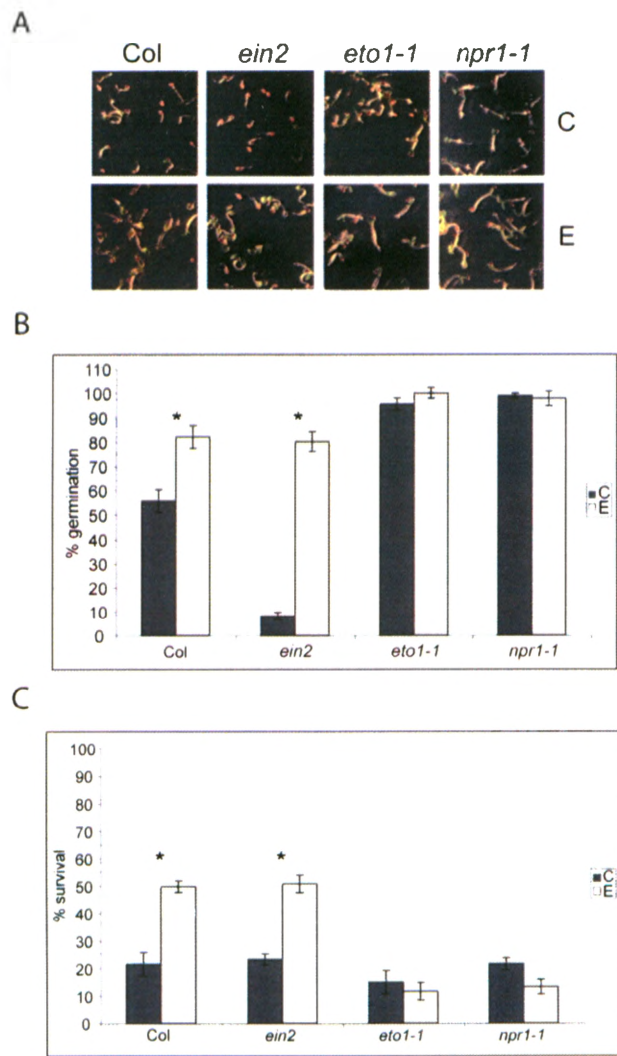

Col
C
E
PR-1
PDF1.2
RD22
GST1
DWF4
ACTIN

**Figure 3.5** EBR effects on inhibition of germination by NaCl in WT and mutant seedlings. **A)** WT (Col), *ein2*, *eto1-1* and *npr1-1* were allowed to germinate on a nutrient medium containing 150 mM NaCl in the absence (C) or presence of 1 μM EBR (E). Photographs of the seedlings were taken 3 days after imbibition. **B)** Percentage of seeds germinated was calculated by counting the number of seedlings with emerged cotyledons at 3 days after imbibition. **C)** Percentage of seedlings surviving on 150 mM NaCl was calculated by counting the number of seedlings that showed true leaves and green colour at 20 days after imbibition. All experiments were performed in triplicates with n > 30. Error bars represent SE of mean for three replicates. The threshold of significance as analyzed by student's t-test is indicated above the histograms (*$p$ <0.01).


A
Col
ein2
eto1-1
npr1-1
C
E
B
% germination
110
100
90
80
70
60
50
40
30
20
10
0
Col
ein2
eto1-1
npr1-1
C
E
C
% survival
100
90
80
70
60
50
40
30
20
10
0
Col
ein2
eto1-1
npr1-1
C
E

of EBR (Figure 3.5A and B). WT seeds showed germination rates of 55% and > 80% on 150 mM NaCl in the absence and presence of EBR, respectively, while *ein2* seeds had germination rates of ~10% and 80% under the same conditions (Figure 3.5B). Seeds of *eto1-1* and *npr1-1* germinated at similar rates (> 90%) on 150 mM NaCl in the absence or presence of EBR (Figure 3.5B). However, despite the good germination efficiency on salt, the average survival rates of *eto1-1* and *npr1-1* on salt were only 15% and 22%, respectively (close to WT), but unlike WT, survival of these mutant seedlings could not be rescued by EBR treatment (Figure 3.5C). These results indicate that NPR1 has a role in salt stress and that BR effects on seedling survival under salt stress require a functional NPR1.

## 3.4 DISCUSSION

In the present study we focused on understanding BR interactions with other stress hormones in mediating increase in stress tolerance mainly because 1) BR is known to interact with other plant hormones in regulating plant developmental processes, and 2) multiple hormone signaling pathways play a role in acquisition of stress tolerance. We evaluated a subset of signaling, biosynthetic and constitutively active mutants of ABA, ET, JA and SA for thermo and salt tolerance in untreated and BR-treated states to assess the importance of these hormones in BR-mediated increase in stress tolerance of Arabidopsis seedlings. Here we demonstrate that NPR1, a protein well recognized for its role in SA-mediated systemic acquired resistance (SAR) and cross-talk inhibition of JA-mediated defense responses, also has a role in BR-mediated stress tolerance.

### *3.4.1 Thermotolerance defects of hormone mutants and BR effects*

SA, and more recently JA, has been linked with thermotolerance. In case of SA genotypes, it is known that *npr1-1* is compromised in basal thermotolerance, while *cpr5-2* has greater thermotolerance than WT (Clarke et al., 2004; Larkindale et al., 2005). Recently it was demonstrated that a JAR1-dependent pathway is also required for basal thermotolerance (Clarke et al., 2009). Using a collection of genotypes with basal thermotolerance either lower or higher than WT, we found that EBR treatment could significantly increase the basic thermotolerance of these genotypes and that this increase

was comparable to the increase in WT. An exception to this result within the SA, JA and ET genotypes was *npr1-1*, indicating that a functional NPR1 is required for full manifestation of BR's effects. Although collectively our data seems to suggest that BR is not critically dependent on SA levels and JA signaling for its anti-stress effects, further confirmation is required with genotypes such as *Nahg* and *coi1* to make an unequivocal claim. Even if BR works to some extent independently of other hormones in conferring heat tolerance, the mere fact that ABA, BR, ET, JA and SA all play a role in thermotolerance of Arabidopsis plants suggests that there must be some redundant and some specific events in the mechanisms by which these hormones produce their effects. With the exception of BR where some information has been obtained (Dhaubhadel et al., 1999; 2002; Kagale et al., 2007), molecular changes mediated by ABA, ET, JA and SA that lead to thermotolerance are largely unknown. In case of ET, the Ethylene Response Factor Protein, JERF3, has been demonstrated to activate the expression of oxidative genes, resulting in decreased accumulation of ROS and, in turn, enhanced adaptation to drought, freezing, and salt in tobacco (Wu et al., 2008). A similar role for JERF3 can be envisioned in response to HS. A number of ABA-regulated genes have been implicated in drought tolerance (Zhu, 2002). Recent functional characterization of the ABA-regulated ERD10 and ERD14 indicated that these proteins could prevent the heat-induced aggregation and/or inactivation of various enzyme substrates (Kovacs et al., 2008). Thus, induction of genes functionally similar to molecular chaperones by ABA during HS may help combat the denaturing stress effects of HS.

Previous studies involving treatment with exogenous ABA (Larkindale and Knight, 2002), *aba* and *abi* mutants (Larkindale et al., 2005), and high ABA producing lines (Ristic and Cass, 1992), have demonstrated the positive effects of ABA on thermotolerance. Under our experimental conditions the differences in the survival rates of untreated WT and *aba1-1* and *abi1-1* mutant seedlings were not striking, but the most pronounced effects of EBR with respect to survival within this set of plants was seen in *aba1-1* seedlings (Figure 3.1B), indicating that endogenous ABA levels suppress BR effects. This notion is supported further by higher accumulation of hsp90, a representative of the hsp families of proteins that are known markers of thermotolerance, in *aba1-1* seedlings as compared to WT (Figure 3.2B). It should be noted that neither SA,

nor ABA or BR mutants are compromised in hsp accumulation (Larkindale et al., 2005; Kagale et al., 2007; present study). We have previously demonstrated that treatment with exogenous EBR can significantly increase hsp accumulation in *B. napus* (Dhaubhadel et al., 1999; 2002), but that this effect in Arabidopsis is subtle (Kagale et al., 2007). Thus, the clear enhancement of hsp accumulation in response to EBR during HS in *aba1-1*, but not in WT (Figure 3.2B), confirms that endogenous ABA levels suppress BR effects in WT even under stress conditions. While this work was under preparation, a study describing ABA inhibition of BR signaling was reported whereby it was shown that ABA increases the expression levels of BR biosynthesis gene *DWF4* and *CPD* to a greater extent in *aba1* than in WT, reinforcing the idea that ABA inhibits BR signaling in WT and that this inhibition is relieved in the *aba1* background (Zhang et al., 2009).

Despite an antagonistic relationship between BR and ABA, data from our microarray experiment suggest that ABA levels rise in response to HS in Arabidopsis and that this increase may be further augmented by BR treatment (Chapter 4). Indeed, ABA content has been reported to increase in pea leaves in response to HS (Liu et al., 2006) and in response to BR under HS in *C. vulgaris* (Bajguz, 2009). Based on these results it can be speculated that BR augments ABA levels and ABA-related effects during HS (Figure 3.6), but it is only when ABA levels are compromised that the BR effects of enhancing stress tolerance become apparent.

### *3.4.2 NPR1, stress tolerance and BR effects*

Although *npr1-1* seedlings have been shown to be defective in basal thermotolerance, the heat sensitivity of this genotype is not dramatically lower than WT (Clarke et al., 2004; Larkindale et al., 2005). However, in our study the mere 2.4-fold increase in percent survival of *npr1-1* in response to EBR treatment as compared to the 9-fold increase in WT Col following HS (Figure 3.1), nearly no change in oxidative damage levels in *npr1-1* in response to EBR (Figure 3.2A), and the lack of EBR effect in increasing survival of *npr1-1* seedlings on salt (Figure 3.5B), explicitly indicates that a functional NPR1 is required for the manifestation of BR effects on seedling stress tolerance. Two questions arise from these observations; 1) what would be the function of

NPR1 during abiotic stress conditions, and 2) how could NPR1 integrate in the BR pathway?

NPR1 is a redox-controlled transcriptional cofactor, which is key to development of SAR and critical for modulating cross-talk between SA and JA signaling (Spoel et al., 2003). In the absence of stress, NPR1 is maintained in a large complex consisting of intermolecular disulfide bonded oligomers, which upon stress are reduced to an active monomeric state (Mou et al., 2003). The monomeric form interacts with TGA-bZIP transcription factors and activates defense gene expression. ROS is a common signal in plant stress responses to both abiotic and biotic stresses (Apel and Hirt, 2004; Dietz, 2008). The notion that ROS could be a signal for the HS response is derived from studies of activation of heat shock transcription factors (Hsfs). The eukaryotic Hsf1 multimerizes and binds to DNA upon either heating or oxidation with $H_2O_2$ (Ahn and Thiele, 2003). More direct evidence showing that endogenous ROS production is necessary for induction of the HS response comes from the observation that a dominant negative allele of Rac1, the small GTPase necessary for the activation of ROS production by membrane-bound NADPH oxidase (Abo et al., 1991), inhibits the stress-induced activation of Hsf1 (Ozaki et al., 2000). Thus, it is highly likely that ROS production during HS also activates NPR1, leading to gene expression changes critical for thermotolerance. Future studies directed at global gene expression analysis in WT and *npr1* mutant in response to HS and BR as separate and combined treatments should indicate NPR1-dependent molecular changes and help clarify the role of NPR1 in BR-mediated increase in thermotolerance.

The mechanism for how BR could function with NPR1, it is speculated that ROS-activated NPR1 monomers may bind a BR-activated regulator to affect stress-responsive gene expression critical to the survival of seedlings under stress conditions. It is unlikely that NPR1 controls BR signaling via BIN2 and BZR1 given that down-regulation of DWF4 in *npr1-1* was unaffected (Figure 3.3C).

### *3.4.3 Oxidative stress state in cpr5 and BR effects*

The *cpr* (CONSTITUTIVE EXPRESSOR OF *PR* GENES) mutants are characterized with increased concentrations of SA, constitutive expression of the *PR*

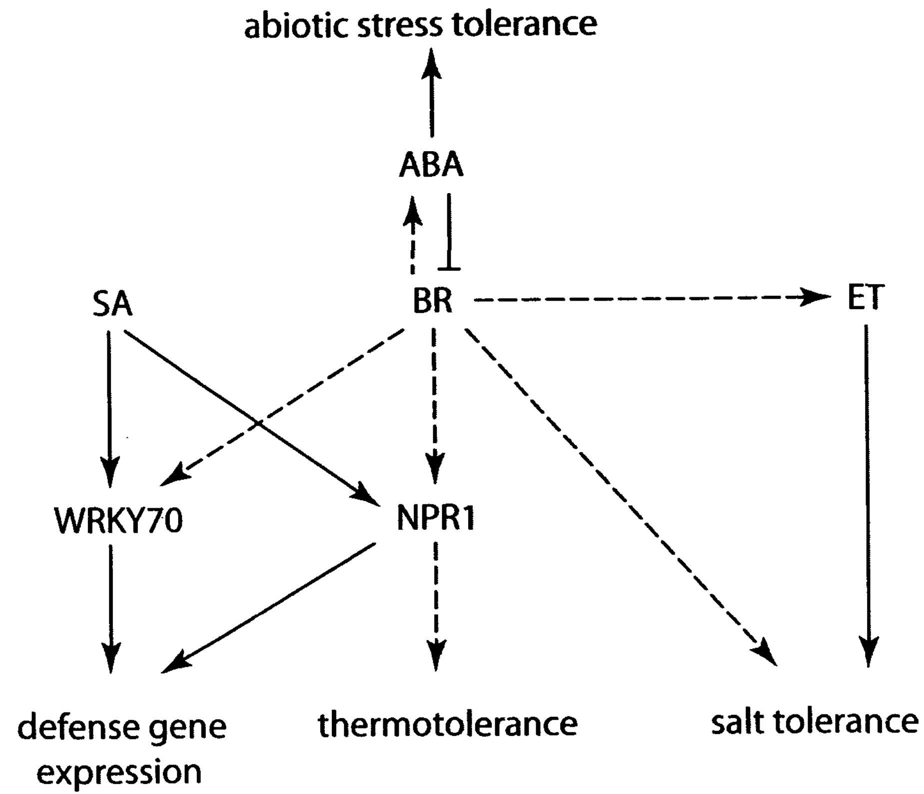
genes, and enhanced resistance to pathogens (Bowling et al., 1994; 1997; Clarke et al., 1998). The *cpr* mutants are in a state of high-cellular oxidative stress state as compared to WT. For example, *cpr1* plants exhibited greater oxidative damage than WT under both normal growth (23$^0$C) and chilling (5$^0$C) conditions (Scott et al., 2004), and molecular changes in *cpr5* such as increased expression of several genes in the ROS gene network, including GSTs, indicate that the cellular redox balance in this mutant is de-regulated (Jing et al., 2008). We also observed higher levels of *GST1* transcript in *cpr5-2* as compared to WT (Figure 3.3B). The high-cellular oxidative stress state of the *cpr* mutants, combined with the constitutively activated SA and JA/ET-mediated pathways (Bowling et al., 1997; Clarke et al., 2000), may trigger not only defense responses against pathogens but also abiotic stress response pathways, leading to greater survival of *cpr5-2* seedlings than WT in response to HS (Figure 3.1B). EBR treatment further enhanced the survival rates of *cpr5-2* seedlings exposed to HS (Figure 3.1B), but had negligible effect on the oxidative damage levels in this mutant (Figure 3.2A), presumably due to the inherent oxidative state of the mutant.

### *3.4.4 BR induces expression of other hormone marker genes*

Up-regulation of *PR-1* by BR in *eds5-1* and *npr1-1* backgrounds (Figure 3.3A), as well as in short-term treatment (Figure 3.4), suggests that BR directly mediates expression of this gene. Interestingly, we found *WRKY70*, a transcription factor acting downstream of NPR1 and involved in the expression of SA-induced *PR* genes (Li et al., 2004), to also be up-regulated by BR in different SA genotypes (Figure 3.3A). Thus, in addition to NPR1, *WRKY70* may be a potential point of cross-talk between SA and BR via which BR may induce a subset of SA-responsive genes. Such a scenario could explain, in part, how BR enhances plant resistance against pathogen infection (Krishna, 2003).

BR could increase the expression of *PDF1.2* in JA mutants, but not to the same extent in the ET-insensitive mutant *ein2* (Figure 3.3A). Thus, at present we favor the possibility that BR effects on *PDF1.2* expression are mediated via the ET pathway. It should be noted that *PR-1* and *PDF1.2* genes are constitutively expressed in *cpr* and *ssi1*

**Figure 3.6** Proposed model for interaction of BR with SA, ABA and ET in mediating stress tolerance. BR positively regulates SA pathway components NPR1 and WRKY70 to mediate thermotolerance and defense gene expression, respectively. ABA suppresses BR effects on thermotolerance, but BR may enhance ABA responses under certain conditions by increasing ABA levels. BR can enhance salt tolerance through an ET-independent pathway or ET-dependent pathway. The dashed arrows represent BR responses, while solid arrows indicate other hormone pathways.


abiotic stress tolerance
ABA
SA
BR
ET
WRKY70
NPR1
defense gene expression
thermotolerance
salt tolerance

mutants of Arabidopsis in an NPR1-independent and SA, JA and ET-dependent manner (Clarke et al., 2000; Nandi et al., 2003), providing precedent for the notion that more than one pathway governs the expression of these genes. The increase in the transcript levels of the ABA-responsive *RD22* gene and the stress-induced *GST1* gene in WT and hormone mutants by EBR, including short-term treatment of WT (Figure 3.4), suggests that these genes may also be primary targets of BR. Taken together, our data indicate that BR has regulatory inputs into the expression of other hormone-responsive genes, which may result from the action of the BR pathway either on the promoters of these genes, or on the regulation of a hormone-signaling component or the biosynthesis of another hormone (Figure 3.6).

### *3.4.5 BR effects on inhibition of seed germination by salt*

We have previously shown in *B. napus* that EBR helps to overcome inhibition of seed germination by salt (Kagale et al., 2007). To study the involvement of BR and other hormones in salt tolerance, we screened WT, *npr1-1, ein2* and *eto1-1* seeds for germination on 150 mM salt. The *ein2-5* mutant has been found to be hypersensitive to salt (Wang et al., 2007) and *ein2-1* displayed sensitivity to heat and osmotic stress (Suzuki et al., 2005). Clearly, EIN2 is an important node for interaction of stress and hormonal signaling pathways. The fact that EBR could rescue hypersensitivity of *ein2* to salt (Figure 3.5A and B) and increase survival rates of *ein2* seedlings following HS (Figure 3.1) with effects paralleling those in WT, unambiguously indicates that BR can bypass ET signaling in conferring stress tolerance in Arabidopsis.

We included *npr1-1* and *eto1-1* in the salt stress study due to the hypersensitivity of these genotypes to HS even in presence of BR. Interestingly, while both *npr1-1* and *eto1-1* were insensitive to salt during germination, both genotypes had survival rates comparable with those of WT and *ein2* in the absence of EBR, and notably lower survival rates than WT and *ein2* in the presence of EBR (Figure 3.5C). These results further endorse a crucial requirement of functional NPR1 in BR-mediated increase in stress tolerance and suggest further explorations of the roles of NPR1 in abiotic stress. The possibility that NPR1 mediates defense responses against abiotic stresses has been suggested in two recent reports (Quilis et al., 2008; Yasuda et al., 2008).

In summary we have demonstrated that 1) the BR-mediated increase in stress tolerance is independent, at least in part, from other hormone signals, 2) NPR1 is a critical component of BR-mediated effects on thermo and salt tolerance, 3) ABA inhibits BR effects in abiotic stress responses, and 4) several hormone-responsive genes are also BR-responsive. These findings point to possible cross-talk of BR with SA, ET and ABA signaling pathways in mediating stress responses, as depicted in Figure 3.6.

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# CHAPTER 4

## Identification and characterization of genes involved in brassinosteroid-mediated stress tolerance

# CHAPTER 4

## 4.1 INTRODUCTION

Brassinosteroids (BRs) are a group of plant steroidal hormones that regulate a wide range of physiological responses in plants, including cell elongation, photomorphogenesis, xylem differentiation, seed germination, (Sasse, 2003) and stress responses (Khripach et al., 2000; Krishna, 2003). Although the growth-promoting properties of BRs were recognized in the early 1970s, the first genetic evidence to suggest that BRs are essential for proper plant development came with the isolation of the BR-deficient mutants *det2* and *cpd* (Li et al., 1996; Szekeres et al., 1996). Numerous other Arabidopsis BR-deficient and BR-insensitive mutants, displaying phenotypic alterations such as dwarfism, small dark-green leaves, a compact rosette structure, delayed flowering and senescence, and reduced fertility (Sasse, 2003), were instrumental in the identification of BR signaling components and in understanding to some extent how BR regulates gene expression (Vert et al., 2005; Gendron and Wang, 2007 ).

BR is perceived at the cell surface by BRI1 (BRASSINOSTEROID-INSENSITIVE 1), a plasma membrane localized leucine-rich repeat (LRR) receptor-like kinase (RLK) (Li and Chory, 1997; He et al., 2000). BR binding to BRI1 induces a series of biochemical events, such as autophosphorylation of BRI1 in its C-terminal domain, dissociation of an inhibitory protein, BKI1 (BRI1 KINASE INHIBITOR 1), and association of BRI1 with another LRR-RLK, BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) (Wang and Chory, 2006; Nam and Li, 2002). Other known components of the BR signaling pathway include the glycogen synthase kinase-3, BIN2 (BRASSINOSTEROID-INSENSITIVE 2), that acts downstream to BRI1-BAK1 recpetor complex and negatively regulates the transcription factors BZR1 (BRASSINAZOLE-RESISTANT 1) and BES1 (*bri1*-EMS-SUPPRESSOR 1) by phosphorylating them (Li and Nam, 2002; Vert and Chory, 2006), while the phosphatase BSU1 (*bri1* SUPPRESSOR 1) positively regulates BR signaling possibly by dephosphorylating BZR1 and BES1 (Mora-García et al., 2004). Neither BIN2 nor BSU1 has been shown to interact with BRI1; thus, how BR signal is transmitted to these downstream proteins is

currently not known. Recently, quantitative proteomic studies of BR-responsive proteins have led to the identification of three BR signaling kinases (BSK1, BSK2 and BSK3), which can be phosphorylated by BRI1 *in vitro* and interact with BRI1 *in vivo* (Tang et al., 2008). The BSK proteins contain a kinase domain at the N-terminal side and tetratricopeptide repeat (TPR) domains at the C-terminus that are known to mediate protein:protein interactions. Although genetic studies point to BSK3 functioning downstream of BRI1 (Tang et al., 2008), it remains to be seen if BIN2 is a direct downstream target of a BSK protein(s). BIN2-catalyzed phosphorylation likely inhibits BZR1 and BES1 functions through targeted degradation, reduced DNA binding, and cytoplasmic retention through interaction with 14-3-3 proteins (Gampala et al., 2007; de Vries, 2007). BRI1 binding to BR inactivates BIN2 and activates BSU1, resulting in the activation and nuclear accumulation of BZR1 and BES1. Activated BZR1 and BES1 directly bind the promoters of BR-regulated genes to affect their expression (He et al., 2005; Yin et al., 2005). The modes of action of BZR1 and BES1 are currently known for only a limited set of BR-responsive genes. BZR1 binds to the CGTG(T/C)G motif found in the promoters of BR biosynthetic genes, *CPD* and *DWF4,* to suppress their expression (He et al., 2005), while BES1 binds to the CANNTG motif (E box) in the *SAUR-AC1* promoter to activate gene expression (Yin et al., 2005). In view of the number of physiological processes that BRs regulate, it was hypothesized that BZR1 and BES1 heterodimerize with other transcriptional factors to regulate transcriptional processes. Indeed, BES1 has been demonstrated to interact with BIMs (BES1-INTERACTING MYC-LIKE PROTEINS), leading to enhanced binding of BES1 to the *SAUR-AC1* promoter (Yin et al., 2005), MYB30 (MYB DOMAIN PROTEIN 30), a transcription factor that acts as a positive regulator of the hypersensitive cell-death response (Li et al., 2008), and the jumonji (Jmj) domain-containing proteins ELF6 (EARLY FLOWERING 6) and REF6 (RELATIVE OF EARLY FLOWERING 6) that are involved in regulating flowering time (Yu et al., 2008). ELF6 and REF6 are recruited to BR target gene promoters by BES1 and possibly modify gene expression through histone modifications. Since Jmj domain-containing histone demethylases are involved in many developmental processes and diseases, it is probable that recruitment of these proteins by BES1 is one of the ways by which BR affects diverse biological processes.

Numerous BR-regulated genes have been identified by genome-wide microarray analyses (Goda et al., 2002; Müssig et al., 2002; Yin et al., 2002). Although similarities in the broad functional categories were observed, these studies showed a little overlap in the genes identified. A high confidence list of BR-regulated genes was generated and a comparative analysis of the gene lists from all these studies was performed revealing the consistent BR responses (Vert et al., 2005). The majority of BR-responsive genes were associated with cell wall biosynthesis and loosening, cytoskeleton formation, phytohormone synthesis and signaling. A close association of the BR and Auxin genomic responses was also revealed (Vert et al., 2005). These genes clearly correlate with the known physiological responses of BRs involving plant growth and development.

Similar to the growth-promoting effects, the stress protective properties of BRs were noted in several preliminary studies, although the associated variability in the efficacy of BRs to promote stress tolerance weakened the impact of the results, which likely contributed to the relatively slower pace of progress made in this direction. It is believed that the mode of BR application (seed soak, root soak or foliar spray), as well as the developmental stages at which BR was applied, were the primary reasons for the inconsistency in the results of earlier studies. Recent attempts in our lab to study BR effects on plant stress responses under standardized conditions have indeed yielded reproducible effects and the role of BRs in plant stress responses has been confirmed (Divi and Krishna, 2009; Dhaubhadel et al., 1999; 2002; Kagale et al., 2007; Koh et al., 2007). BR promotes tolerance in plants to a wide range of stresses, including heat, cold, drought and salinity, and this increase is generally correlated with higher expression of stress marker genes, such as *heat shock protein* (*hsp*) genes, *RESPONSIVE TO DESSICATION29A (RD29A)*, and *EARLY RESPONSIVE TO DEHYDRATION10* (*ERD10)* (Dhaubhadel et al., 1999; Kagale et al., 2007), indicating that increased expression of stress-responsive genes is responsible, in part, for the higher stress tolerance in BR-treated plants. However, a thorough understanding of mechanisms by which BR controls plant stress responses and regulates the expression of stress response genes is lacking.

To identify additional BR-induced gene expression changes in *Brassica napus* seedlings, the differential display–reverse transcription PCR technique was used. Several

cDNAs showing significant changes in expression levels in response to BR were isolated and identified as genes encoding a mitochondrial transcription termination factor (mTERF)-related protein, glycine-rich protein 22 (GRP22), myrosinase, and 3-ketoacyl-CoA thiolase (Dhaubhadel and Krishna, 2008). Transcripts of mTERF-related protein, GRP22, and myrosinase were present at approximately 2-, 4-, and 6-fold higher levels, respectively, in treated seedlings before heat stress (HS), whereas those of 3-ketoacyl-CoA thiolase rose to higher levels in treated seedlings during exposure to HS. These results indicate that BR treatment in *B. napus* leads to substantial changes in the expression levels of genes involved in a variety of physiological responses, either before or during HS exposure (Dhaubhadel and Krishna, 2008). Our previous studies also revealed that, while *B. napus* seedlings treated with 24-epibrassinolide (EBR), a BR, accumulated significantly higher levels of hsps in response to HS (Dhaubhadel et al., 1999), hsp transcript and protein levels were comparable between EBR-treated and untreated wild-type (WT) Arabidopsis seedlings during HS (Kagale et al., 2007). However, EBR treatment increased the basic thermotolerance in both *B. napus* and Arabidopsis (Dhaubhadel et al., 1999; Kagale et al., 2007). These results highlighted the varied gene expression changes between the two plant species in response to BR and stress. Further investigations in *B. napus* revealed that BR treatment modulates the translational machinery, which leads to higher hsp synthesis during stress, more rapid resumption of cellular protein synthesis following HS and a higher survival rate (Dhaubhadel et al., 2002). To identify BR responses before and during HS conditions on a global scale, we reverted to using Arabidopsis due to the many resources available for this experimental system.

The EBR and HS treatment conditions in Arabidopsis under which the stress alleviating effects of BR can be studied in a reproducible manner have been established previously (Kagale et al., 2007). In the present study, analysis of global gene expression in EBR-treated and untreated Arabidopsis before, during and after HS was undertaken to identify genomic BR responses that help in modulating plant stress tolerance. The transcriptional changes in response to EBR under no-stress (0 h) and HS (1 h and 3 h at $43^0$C); and transcriptional changes in response to HS (1 h and 3 h) were analyzed. This allowed a comparison of transcriptional changes in response to EBR alone or HS alone,

as well as the effect of EBR on HS response (EBR+HS). The trends of transcript changes in response to EBR, HS and EBR+HS can be classified into two types: 1) those that were regulated by EBR alone under non-stress and HS conditions; and 2) those that were primarily HS-regulated and their expression was influenced by EBR in the absence of stress and further augmented in response to stress. Investigation of the differentially expressed genes revealed that the second largest category of genes affected by BR consists of genes previously linked with abiotic and biotic stress tolerance. Analysis of these genes using publicly available microarray datasets revealed that there exists considerable overlap with genes responsive to abscisic acid (ABA) and jasmonic acid (JA). Preliminary investigations of knockout (KO) mutants of a subset of genes identified as being affected by both EBR and stress have revealed new stress-related genes in Arabidopsis. Altogether, these results have shed light on a macro scale on how BR may promote stress tolerance in plants and how these effects may be integrated with its effects on plant growth and development. Such an understanding is important when contemplating changes of plant architecture, productivity, or sustainability through manipulation of BR levels.

## 4.2 MATERIALS AND METHODS

### *4.2.1 Plant growth and stress treatment for microarray*

Arabidopsis ecotype Columbia, was used for microarray experiments. Growth and HS conditions were as described previously (Kagale et al., 2007). Briefly, seedlings were grown for 21 days on a nutrient medium supplemented with either 1 µM EBR or 0.01% ethanol (solvent for EBR). HS was given to 21-day-old seedlings by exposing seedlings to $43^0$C for 1 h and 3 h. For recovery, seedlings were transferred to $22^0$C following 3 h of HS to recover for a period of 6 h. Plant tissue above the medium was collected from unstressed (0 h), heat stressed (1 h and 3 h) and recovering (6 hR; 6 h at $22^0$C following 3 h of HS) seedlings and quick-frozen. For each treatment, plant material was pooled from at least 30 seedlings to minimize the effect of variation among individual plants and quick frozen.

### *4.2.2 RNA extraction and microarray hybridization*

RNA was isolated from frozen tissue of three independent biological replicates using Plant RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). Plant growth, stress treatments and RNA isolation were performed by Sateesh Kagale. Quality check, probe preparation and hybridization were performed at the London Regional Genomics Centre (LRGC; Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca). RNA quality was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) using RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA, USA) and analysis was performed by the Degradometer (www.dnarrays.org). Probe preparation and array hybridization were performed according to Affymetrix GeneChip Techincal Analysis Manual (Affymetrix, Santa Clara, CA, USA). Biotin-labeled complimentary RNA (cRNA) was prepared from 10 μg of total RNA. Labeled cRNA (10 μg) was then hybridized to Arabidopsis ATH1 GeneChips for 16 h at $45^0$C. After hybridization the GeneChips were stained with Streptavidin-Phycoerythrin, followed by washing with Anti-Streptavidin antibody solution and staining with Streptavidin-Phycoerythrin for second time. All the liquid handling for washes was performed in GeneChip Fluidics station 450. The GeneChips were then scanned with the Affymetrix GeneChip Scanner 3000.

### *4.2.3 Microarray data analysis*

Normalization and expression analysis were performed using Genespring GX 10 software (Agilent Technologies Inc., Palo Alto, CA, USA). Expression estimates for each gene were generated using gcRMA (Wu et al., 2004). Signal intensities were normalized to the baseline median across all the chips. To identify differentially expressed genes, the $\log_{10}$ normalized signals were subjected to two-way analysis of variance (ANOVA). In all analyses a *P*-value of 0.05 was selected. The Benjamini and Hochberg false discovery rate (FDR) multiple testing correction (Benjamini and Hochberg, 1995) was applied to minimize the detection of false positives. All genes that changed ≥ 2-fold in expression between treatments were considered as differentially expressed.

*4.2.4 Hierarchical clustering and distribution of functional categories*

Following normalization, genes differentially expressed by EBR, HS and EBR+HS were subjected to hierarchical clustering in Genespring GX 10 software (Agilent Technologies Inc., palo Alto, CA, USA). For each cluster the gene ontology (GO) term and promoter motif analyses were performed using Athena data analysis suite (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/analysis_select.pl). Assignment of genes into biochemical pathways and biological processes were done using MapMan pathway analysis software (Thimm et al., 2004) and according to GO slim terms (biological processes) at The Arabidopsis Information Resource (TAIR) website (http://www.arabidopsis.org/tools/bulk/go/index.jsp). Genes overlapping between treatments and genes unique to treatments were identified by Venn diagram analysis in Genespring software.

*4.2.5 In Silico analyses of candidate genes*

Public resources like National Center for Biotechnology Information (NCBI), TAIR, Munich Information Center for Protein Sequences (MIPS) and InterPro (http://www.ebi.ac.uk/interpro/index.html) and its member databases were used to mine the information on interesting genes. The NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) was used to predict the putative domains and the MyDomains-Image Creater tool of PROSITE (http://ca.expasy.org/cgi-bin/prosite/mydomains/) was used for creation of domain features. The data on abiotic stress- and hormone-responsive gene expression profiles were generated using AtGenExpress Visulaization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp).

*4.2.6 Phylogenetic analysis*

Sequences related to BR RESPONSIVE RECEPTOR LIKE KINASE (BRRLK) were identified by searching the reference protein (refseq_protein) databases of Arabidopsis using PSI-BLAST (Position-Specific Iterated BLAST) algorithm at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The first 50 sequences retrieved were aligned by the multiple sequence alignment program CLUSTALW 2.0 (Larkin et al., 2007) using default settings. The aligned sequences were used to generate neighbor-joining tree to

infer their possible relationships using MEGA4 phylogenetic software package (Tamura et al., 2007).

#### *4.2.7 RNA isolation and quantitative RT-PCR*

To validate the expression profiles obtained from microarray experiment, the relative expression of 9 selected genes was measured under similar conditions using real-time PCR. Two batches of RNA from microarray experiment and two additional batches of RNA from separate experiments were used. For the new sets, RNA was extracted from the frozen plant tissue using the SV Total RNA Isolation System (Promega, Madison, WI). Total RNA (3 μg) was reverse transcribed using the oligo $(dT)_{18}$ primer and Super Script First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR reactions were performed using SYBR-Green I (Invitrogen, Carlsbad, CA) at 0.1 x concentration, and Rotor Gene-3000 (Corbett Research, Sydney, Australia) instrument to monitor cDNA amplification. Reactions were performed in duplicate for each biological replicate with an initial denaturation step at $94^0$C for 4 min followed by various cycles of denaturation (15 s at $94^0$C), annealing (30 s at $56^0$C) and extension (30 s at $72^0$C and 15 s at $83^0$C). Gene sequences available in GenBank were used to design primers for all the 9 genes (Appendix 4.1). GenScript primer design tool (https://www.genscript.com/ssl-bin/app/primer) was used and expression level of each gene was normalized to *UBIQUITIN10* (*UBQ10*).

#### *4.2.8 Screening of Homozygous plants with T-DNA insertions*

For the selected genes, the *Arabidopsis thaliana* Integrated Database (ATIDB; http://atidb.org/) and the SIGnal database (http://signal.salk.edu) were searched for available T-DNA insertion lines. Lines most likely to cause loss of gene function were obtained from Arabidopsis Biological Resource Centre (ABRC; Ohio state university, Columbus, OH) (Alonso et al., 2003). Homozygous knockout line for *WRKY17* (*wrky17.1*) gene was a kind donation from Dr. Thomas Kroj (Laboratory of Plant-Microorganism Interactions, France).

Plant DNA from young leaves was extracted using CTAB method according to Doyle and Doyle (1990). Homozygous insertion of the lines was confirmed by

conducting PCR using T-DNA left border specific primers LBal (5' GCGTGGACCGCTTGCTGCAACT) or LBb1.3 (5' ATTTTGCCGATTTCGGAAC) and the T-DNA flanking primers (LP and RP) for each gene generated by SIGnal T-DNA primer design tool (http://signal.salk.edu/tdnaprimers.html; Appendix 4.2). The amplification conditions used were as follows: $94^0$C for 5 min; 35 cycles of $94^0$C for 1 min, $55^0$C for 45 sec and $72^0$C for 1 min; final extension at $72^0$C for 5 min.

The knockout lines were further confirmed by RT-PCR. RNA isolation and cDNA synthesis from 10-day-old insertion lines and WT plants were performed as described above for qRT-PCR analysis. Primers specific to cDNA were designed by using the sequences available from GenBank (Appendix 4.3). The amplification conditions were as follows: $94^0$C for 5 min; 30 cycles of $94^0$C for 1 min, $55^0$C for 45 sec and $72^0$C for 1 min; final extension at $72^0$C for 5 min. The amplification of *ACTIN* gene was tested in all the lines as a control. The presence of transcript for every gene in WT plants was tested simultaneously.

### *4.2.9 Phenotypic analysis*

#### *4.2.9a Plant material and growth conditions*

The *wrky33* (SALK_006602), *wrky17.1* (SALK_076337c), *acp5* (SALK_097940), and *brrlk* (SALK_067463c), T-DNA lines were all from Columbia (Col) background. The WT (Col) was used in all experiments as control. Seed were surface sterilized in 75% ethanol for 30 sec followed by 20% bleach for 15 min and plated on 1X Murashige and Skoog medium (Sigma, St. Louis) supplemented with B5 vitamins, 1% (w/v) agar and 0.5% sucrose. The plates were then kept for 3 days in the dark at $4^0$C to encourage synchronized germination and then transferred to $22^0$C with a 16/8 h photoperiod (80 $\mu$E $m^{-2}$ $s^{-1}$) to allow germination and growth.

#### *4.2.9b Heat stress treatments*

Seedlings grown for 5 days in the above conditions were subjected to HS at $44^0$C for 90 min and returned to growth chamber for recovery. The number of seedlings surviving is scored after 7 days of recovery. Seedlings that showed bleaching were

considered dead and those with green leaves were considered surviving. Values in Figure 4.17B are average percentages of data obtained in three biological replicates.

*4.2.9c Salt stress treatments*

Salt treatment was given by including 150 mM NaCl in the medium. All plates were kept for 3 days in the dark at $4^0$C and then transferred to $22^0$C. Seeds were allowed to germinate in the presence (150mM NaCl) or absence of salt. Percent survival was calculated by counting the number of seedlings that showed true leaves and green colour at 15 days after imbibition. Values in Figure 4.18B are average percentages of data obtained in three biological replicates.

## 4.3. RESULTS

### *4.3.1 Identification of genes significantly changed in response to EBR, HS and EBR+HS*

We have previously demonstrated that treatment with EBR of Arabidopsis seedlings increases basal thermotolerance of seedlings, as compared to untreated seedlings, in a consistent and reproducible manner (Kagale et al., 2007). To assess the effects of EBR on global gene expression under no-stress and stress conditions, gene expression profiles were generated from EBR-treated (E; 1µM EBR for 21 days) and untreated (C; 0.01% ethanol for 21 days) Arabidopsis seedlings under no-stress (0 h), HS (1 h and 3 h) and recovery from HS (6 hR) conditions (Figure 4.1A). Samples from three biological replicates were hybridized to ATH1 Affymetrix genome arrays. The variability between independent experiments was analyzed by Principal Component Analysis (PCA) in Genespring software, version GX 10. The average raw signal intensities of all probe sets from the three replicates were plotted on X, Y and Z axes, respectively, to generate 3-D scatter plots. The large level of overlap of the data from three different replicates for each treatment, as well as of clustering of different treatment data into distinct groups, indicate that there was good correlation between the replicates (Figure 4.1B). In the entire experiment, 1823 genes were significantly changed with a $p$-value of $< 0.05$. Out of these 273, 239, 256 and 146 genes showed $\geq$ 2-fold change by

EBR at 0 h, 1 h, 3 h and 6 hR, respectively, and 225, 230 and 277 genes were changed ≥ 2-fold by HS at 1 h, 3 h and 6 hR, respectively.

### *4.3.2 Validation of microarray data using quantitative RT-PCR*

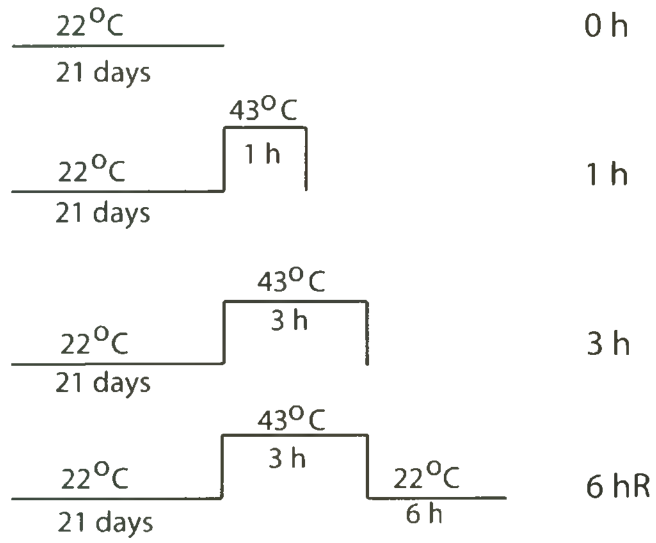
The reliability of the microarray data was evaluated by qRT-PCR for 20 genes differentially expressed in at least one time point of the entire experiment. A high degree of correlation ($r$ = 0.92) between the log ratios from both techniques was observed, indicating good consistency between the two methods (Appendix 4.4). A comparison of the relative expression levels of 9 genes by qRT-PCR and microarray analysis is shown in Figure 4.2. Analysis by qRT-PCR of 11 additional genes by Tawhidur Rahman also showed good correlation between the patterns of expression by the two methods.

### *4.3.3 Expression patterns of genes significantly changed in response to EBR, HS and EBR+HS*

To determine the expression patterns in response to EBR, HS and EBR+HS, a hierarchial clustering analysis was performed using Euclidean distance and average linkage in Genespring. Data from 0 h, 1 h and 3 h time points from EBR-treated (E) and untreated (C) conditions were examined in detail. In this analysis, gene expression by EBR alone (0E), HS alone (1C and 3C) and EBR+HS (1E and 3E) treatments was compared to 0C. Genes significantly changed by ≥ 2-fold ($p < 0.05$) under the different conditions as compared to 0C could be broadly divided into 9 distinct clusters on the basis of expression patterns (Figure 4.3). Genes within Cluster 1 were highly up-regulated by EBR in the absence of stress (0E), but down-regulated by HS (1C and 3C) and EBR+HS (1E and 3E). Cluster 2 genes were down-regulated under all conditions (EBR, HS and EBR+HS), with maximal down-regulation under EBR+HS. Genes within Cluster 3 were specifically up-regulated by HS and down-regulated by EBR and EBR+HS. Cluster 4 includes genes marginally down-regulated by EBR alone, highly down-regulated by HS, and maximally down-regulated by EBR+HS. Cluster 4 genes are distinct from cluster 2 genes in that they had high level of expression under normal conditions, but were down-regulated by other conditions. Also, the effect of EBR was

**Figure 4.1** Overview of microarray experiment. **A)** Growth and heat stress (HS) conditions employed for microarray study. Seedlings grown for 21 days in the presence or absence of 1μM EBR were subjected to either no-stress (0 h) or HS by exposing seedlings to $43^0$C for 1 h and 3 h. For recovery, seedlings were transferred to $22^0$C following 3 h of HS to recover for a period of 6 h (6 hR). For each treatment, plant material pooled from at least 30 seedlings and from three independent biological replicates was hybridized to ATH1 Affymetrix genome arrays. **B)** 3-D scatter plot showing the overlap of data from three different replicates for each time point. The variability between the replicates was analyzed by Principal Component Analysis (PCA) in Genespring software. The average raw signal intensities of all probe sets were plotted on X, Y and Z axes, respectively, to generate 3-D scatter plots. Each color represents a treatment condition, clustering of different treatment data into distinct groups indicate a good correlation between the replicates.

A




B

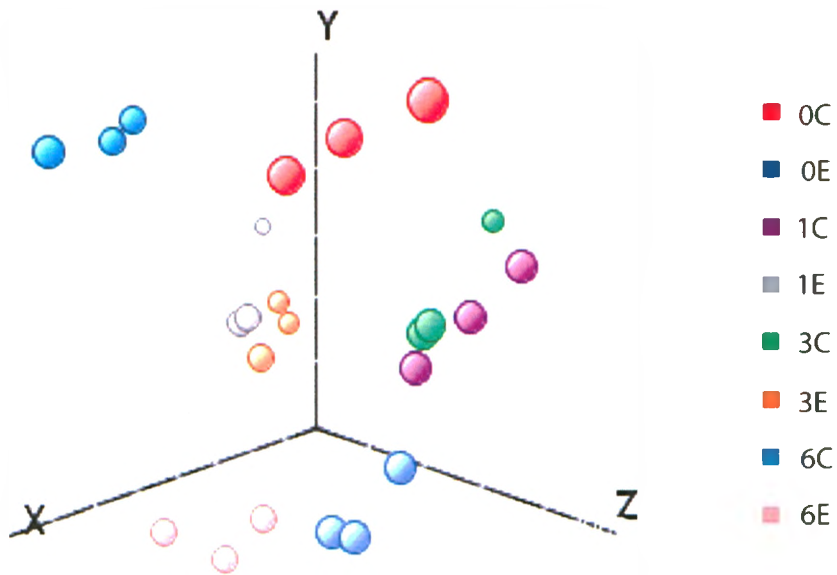
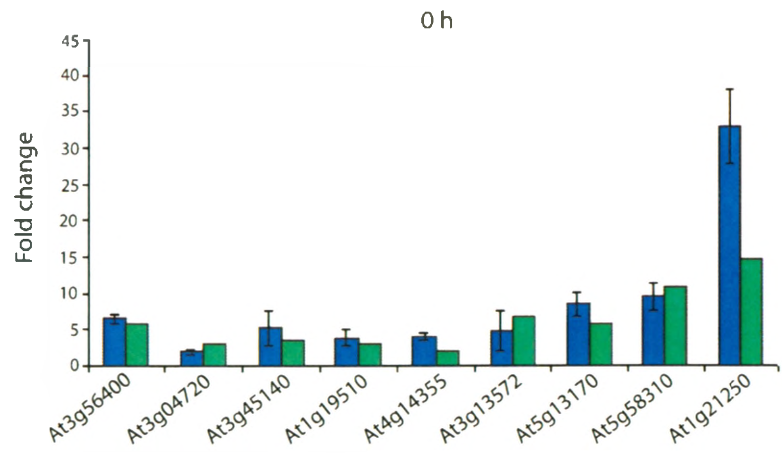


**Figure 4.2** Quantitative RT-PCR (qRT-PCR) analysis for the validation of microarray data. The relative fold change in expression of 9 genes in EBR-treated *vs.* untreated samples at 0 h, 1 h, 3 h and 6 hR time points were determined by qRT-PCR. The fold change values deduced by qRT-PCR were compared to the values obtained from microarray analysis. The signal intensity of each gene was normalized using the *UBQ10* (At4g05320) gene. The Arabidopsis Genome Initiative (AGI) locus identifier of each gene is provided on the X-axis. Error bars indicate standard errors from four biological replicates.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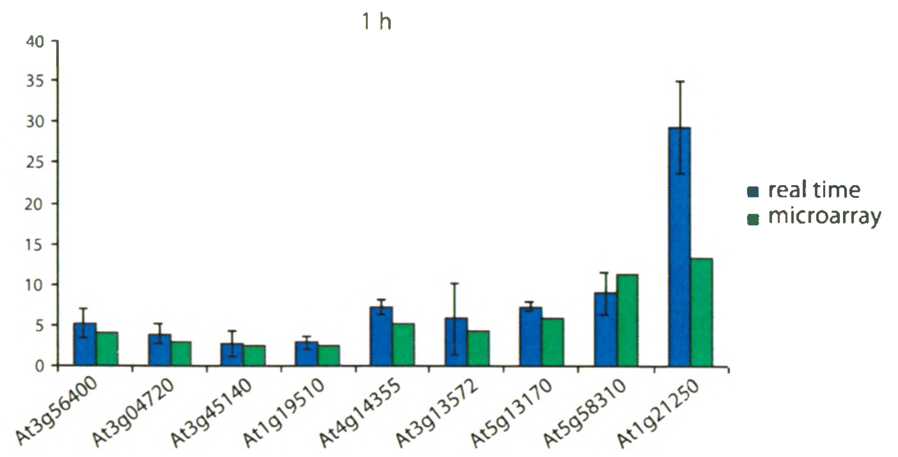
At1g21250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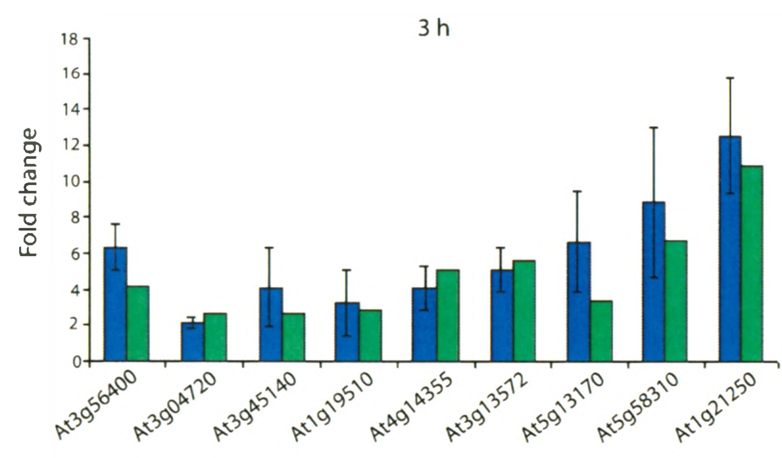
microarray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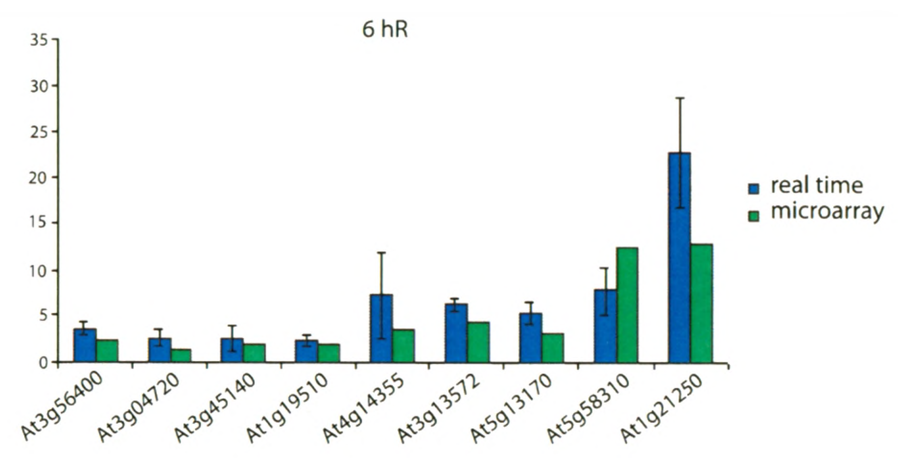
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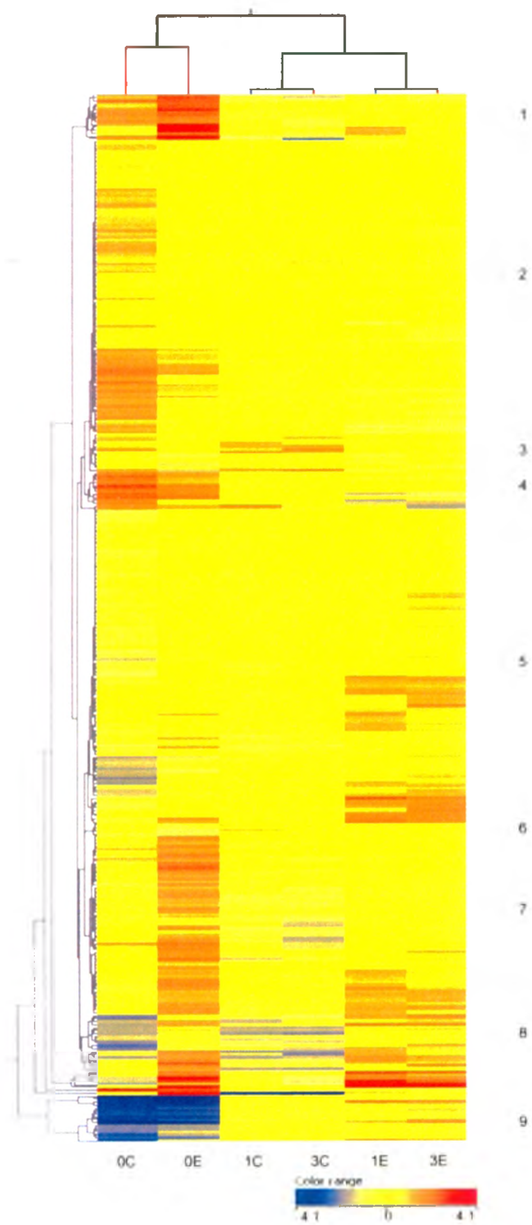
At1g21250
real time
microarray

**Figure 4.3** Hierarchial clustering of 708 genes that were significantly changed ($p < 0.05$) by $\geq$ 2-fold in response EBR alone (0E), HS alone (1C and 3C) and EBR+HS (1E and 3E) treatments as compared to the no-EBR and no-stress (0C) control. Cluster analysis was performed using Euclidean distance and average linkage rule in Genespring. Grouping of genes into 9 major clusters based on expression patterns is indicated. Color scale is indicative of normalized expression level.

U.W.U. LIBRARY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less on cluster 4 genes as compared to cluster 2 genes. Cluster 5 genes were up-regulated by conditions in the following order EBR+HS>EBR>HS. The expression patterns of cluster 2 and cluster 5 indicate HS-specific regulation, with an added effect of EBR on the down- and up- regulation of genes, respectively. The genes in cluster 6 were down-regulated by EBR alone, but up-regulated by HS and only partially constrained by EBR+HS, indicating that cluster 6 genes are predominantly under HS regulation. Genes regulated in opposite directions by EBR and HS were grouped into clusters 7 and 8. These genes were maximally up-regulated by EBR alone and down-regulated by HS. EBR+HS slightly up-regulated cluster 7 genes, whereas cluster 8 genes were highly up-regulated by EBR and EBR+HS. Cluster 9 consists of a unique set of genes that had very low transcript abundance under normal conditions. These genes were highly up-regulated by HS, and even more by EBR+HS. EBR alone had minimal effect on these genes.

### *4.3.4 Gene ontology analysis of genes significantly changed in response to EBR, HS and EBR+HS*

Genes that share similar expression profiles across different conditions are likely to be under common transcriptional regulation. To correlate genes within each cluster with cellular activities and location, each gene cluster was analyzed for significantly enriched Gene ontology (GO) term by using the data-mining tool of Athena database (O'Connor et al., 2005). A $p$-value cut off of $10^{-3}$ was administered. As shown in Table 4.1, the cellular components chloroplast and cytoplasm were over-represented in cluster 2 ($p < 10^{-10}$) and cluster 5 ($p < 10^{-3}$), respectively, and the endomembrane system was over-represented in clusters 1 ($p < 10^{-8}$) and 7 ($p < 10^{-10}$). Several GO terms like cell wall loosening, sexual reproduction, lipid transport and lipid-binding were over-represented in cluster 1, indicating that genes involved in these activities are induced by EBR, but suppressed by HS even in the presence of EBR.

Cluster 8 genes were responsive to EBR under both no-stress (EBR) and HS conditions (EBR+HS), while cluster 9 was responsive to EBR only during HS. Since both ABA- and stress- responsive genes were over-represented in clusters 8 and 9 (Table 4.1), it would appear that BR regulates stress responses both directly and through interaction with ABA pathways.

Genes in cluster 7, which were highly up-regulated by EBR, were enriched in terms like cell wall and JA biosynthesis. Genes in cluster 5, which were up-regulated by EBR, HS and also EBR+HS, were enriched in terms like calcium binding, protein kinase activity, sucrose biosynthesis, glutathione dehydrogenase activity, along with response to stress, wounding and desiccation, indicating mainly regulatory and stress-related roles for these genes. Genes involved in electron transport activity were enriched in cluster 2. GO terms like superoxide metabolism, arsenate reductase (glutaredoxin) activity, thiol-disulfide exchange intermediate activity were predominantly associated with genes in clusters 3 and 6 (Table 4.1).

The overall picture emerging from this analysis is that of BR's involvement in regulating cellular processes related to development, stress and hormone metabolism, with predominant BR effects on the expression of known ABA- and stress-responsive genes. While our study reinforces the known roles of BR in these cellular processes, several novel genes regulated by BR have been uncovered and a strong link of BR with stress-responsive genes has emerged. It remains to be seen whether these BR-regulated genes are under direct or indirect control of BR.

***4.3.5 Identification of regulatory motifs associated with genes in different clusters***

To better define co-expression of genes within clusters, putative promoter regions of sets of co-expressed genes (genome sequences 1500 bp upstream of the transcription start site) were screened for over-represented motifs by using the promoter visualization tool in Athena database as described by O'Connor et al. (2005). A list of significantly enriched transcription factor binding motifs ($p < 10^{-3}$) in each cluster is given in Table 4.2. Interestingly, ABA response element (ABRE)-like binding site motif was over-represented in clusters 7 and 8 that were up-regulated by both EBR and EBR+HS. There was no significant enrichment for any transcription factor site in clusters 1, 3, 4 and 6. Motifs like AtMYB2 BS in RD22, AtMYC2 BS in RD22, MYCATERD1, W-box, ATHB2, and ACGTABREMOTIFA2OSEM, were over-represented in cluster 7; DREB1A/CBF3 and LTRE motifs were enriched in cluster 8; the CARGCW8GAT motif was enriched in cluster 5; and the light-responsive Ibox motif was over-represented in cluster 2, which consists of genes down-regulated by HS, EBR and EBR+HS. The

**Table 4.1** Gene ontology (GO) terms enriched in each cluster of genes significantly changed by EBR, HS and EBR+HS.

| Cluster ID | *p*-value | GO term |
|---|---|---|
| 1 | < 10 - 8 | endomembrane system |
| | < 10 - 3 | purine transporter activity |
| | < 10 - 4 | extracellular |
| | < 10 - 3 | lipid transport |
| | < 10 - 3 | lipid binding |
| | < 10 - 3 | cell wall loosening |
| | < 10 - 3 | ccaat-binding factor complex |
| | < 10 - 3 | carboxylic ester hydrolase activity |
| | < 10 - 3 | sexual reproduction |
| 2 | < 10 - 10 | chloroplast |
| | < 10 - 5 | electron transporter activity |
| | < 10 - 3 | selenium binding |
| | < 10 - 3 | cyclopropane-fatty-acyl-phospholipid synthase activity |
| 3 | < 10 - 5 | phosphoribosylaminoimidazole carboxylase activity |
| | < 10 - 4 | /de novo/ imp biosynthesis |
| | < 10 - 3 | superoxide metabolism |
| | < 10 - 5 | phosphoribosylaminoimidazole carboxylase complex |
| | < 10 - 3 | superoxide dismutase copper chaperone activity |
| 4 | < 10 - 5 | two-component response regulator activity |
| 5 | <10 - 4 | cytoplasm |
| | < 10 - 3 | protein kinase activity |
| | < 10 - 3 | protein serine/threonine kinase activity |
| | < 10 - 3 | protein-tyrosine kinase activity |
| | < 10 - 5 | calmodulin binding |
| | < 10 - 3 | sucrose biosynthesis |
| | < 10 - 3 | protein amino acid phosphorylation |
| | < 10 - 4 | response to stress |
| | < 10 - 3 | response to dessication |
| | < 10 - 3 | response to wounding |
| | < 10 - 3 | hypersensitive response |
| | < 10 - 3 | glutathione dehydrogenase (ascorbate) activity |

| | | |
|---|---|---|
| 6 | < 10 - 8 | cysteine protease inhibitor activity |
| | < 10 - 3 | electron transporter activity |
| | < 10 - 8 | arsenate reductase (glutaredoxin) activity |
| | < 10 - 6 | thiol-disulfide exchange intermediate activity |
| 7 | < 10 - 10 | endomembrane system |
| | < 10 - 3 | cell wall |
| | < 10 - 4 | jasmonic acid biosynthesis |
| | < 10 - 3 | 12-oxophytodienoate reductase activity |
| 8 | < 10 - 6 | lipid transporter activity |
| | < 10 - 4 | response to stress |
| | < 10 - 7 | response to cold |
| | < 10 - 7 | cold acclimation |
| | < 10 - 8 | response to abscisic acid stimulus |
| | < 10 - 4 | hyperosmotic salinity response |
| 9 | < 10 - 4 | lipid transporter activity |
| | < 10 - 4 | response to stress |
| | < 10 - 4 | response to water |
| | < 10 - 4 | response to abscisic acid stimulus |

Table 4.1 contd.

**Table 4.2** Promoter motifs enriched in each cluster of genes significantly regulated by EBR, HS and EBR+HS.

| Cluster ID | *p*-value | Promoter motif |
|---|---|---|
| 2 | < 10 - 3 | Ibox promoter motif |
| 4 | < 10 - 4 | CARGCW8GAT |
| 7 | < 10 - 6 | ABRE-like binding site motif |
| | < 10 - 3 | ACGTABREMOTIFA2OSEM |
| | < 10 - 3 | ATHB2 binding site motif |
| | < 10 - 3 | AtMYB2 BS in RD22 |
| | < 10 - 5 | AtMYC2 BS in RD22 |
| | < 10 - 5 | MYCATERD1 |
| | < 10 - 3 | W-box promoter motif |
| 8 | < 10 - 4 | ABRE-like binding site motif |
| | < 10 - 3 | DREB1A/CBF3 |
| | < 10 - 3 | LTRE promoter motif |
| 9 | < 10 - 4 | ABRE-like binding site motif |

consensus sequence for each motif can be obtained from AtcisDB (http://arabidopsis.med.ohio-state.edu/) and PLACE (http://www.dna.affrc.go.jp/PLACE/) databases. The enrichment of the ABRE-like binding motif in several gene clusters was by far the most notable piece of information, indicating that several ABA-regulated genes are responsive to BR either directly or via cross-talk with ABA.

### *4.3.6 Distribution of genes responsive to EBR, HS and EBR+HS into functional categories*

Genes changing under EBR, HS and EBR+HS were assigned to functional bins according to MapMan pathway annotator (Thimm et al., 2004). The normalized expression values of genes significantly changed under each condition were incorporated into MapMan. Of the 35 bins available in MapMan, significant changes were observed in genes belonging to 10 functional bins. The distribution of genes into selected functional bins and the expression level of individual genes in each category are shown in Figure 4.4. Overall, genes related to cell wall, lipid metabolism, hormone metabolism, redox regulation, stress, RNA, protein, signaling, transport and development were significantly changed. To allow comparisons between expression patterns and functional categorization, the distribution of genes from each functional bin among different clusters is shown (Figure 4.4). It is notable that 50% of the RNA bin genes belong to cluster 2, which includes genes down-regulated under all conditions and maximally under EBR+HS. GO analysis of RNA bin genes from cluster 2 revealed that 48% of these genes were involved in transcription, and approximately 8.7%, 13%, and 8.7% were involved in developmental processes, cell organization/biogenesis, and stress responses, respectively.

The highest proportion of genes in signaling (48% of all genes in the signaling bin) and stress (31%) bins belong to cluster 5, which consists of genes up-regulated by EBR, HS and even more by EBR+HS. This observation highlights BR's involvement in plant stress response, and, presumably cell signaling associated with it. Majority of the genes in the development bin belong to clusters 7 and 8, which includes genes up-regulated by EBR and EBR+HS, but down-regulated by HS. This would be expected given the critical role of BR in plant development. The lipid metabolism and hormone

metabolism bins are enriched with genes from clusters 5 and 7. Since all these clusters are primarily affected by EBR, it can be concluded that lipid metabolism, cross-talk with other plant hormones, and stress response, are categories linked with BR function. Cell wall genes, a category previously linked with BR (Müssig and Altmann, 2003), were also up-regulated by EBR and EBR+HS, but down-regulated by HS alone.

### *4.3.7 Effects of EBR, HS and EBR+HS on protein metabolism*

Earlier we demonstrated that BR promotes protein synthesis during stress conditions by modulating the expression of some of the components of the translational machinery (Dhaubhadel et al., 2002). The present study extends our understanding of BR's effect on protein metabolism through identification of 58 genes related to protein metabolism and regulation that were significantly changed ($p < 0.05$) and classified into groups A to E based on their responses to EBR, HS and EBR+HS (Figure 4.5). Nearly 50% of the genes in group E are related to protein synthesis and protein degradation and these were down-regulated by EBR, HS and EBR+HS. Group D genes, constituting 17% of all genes related to protein metabolism, were up-regulated by all treatments, but mainly by EBR and EBR+HS. Ten out of 15 genes in this group were correlated with post-translational modifications of proteins, while the other 5 were involved in protein degradation, including two aspartate proteases, an ubiquitin E3 ligase and a gene involved in autophagy. It would be interesting to know in the future if the activities of these genes impact stress tolerance.

Genes regulated in opposite directions by EBR and HS were classified under group B. These genes were up-regulated by EBR and EBR+HS, but down-regulated by HS. The 4 genes in group B include a zinc finger (C3HC4-type RING finger) family gene (At5g47070), which is similar to *BRH1 (BRASSINOSTEROID-RESPONSIVE RING-H2)*, two serine carboxy peptidases (*SCPL51*; At2g27920 and *SCPL35*; At5g08260), and a plasma membrane-localized receptor kinase (At5g47070), which undergoes changes in phosphorylation states in response to pathogen elicitors (Benschop et al., 2007).

The group A genes were up-regulated by EBR alone but down-regulated by HS and EBR+HS, indicating major regulation of these genes by HS. This group includes one

gene involved in targeting to secretory pathways (endoplasmic reticulum) and others in protein degradation, including a subtilase, three proteases and an ubiqutin E3 ligase.

### *4.3.8 Genes significantly changed by EBR during no-stress (0 h) and heat stress (1 h and 3 h)*

#### *4.3.8a Identification of 'BR response genes'*

Results described thus far have highlighted changes by EBR alone (0E), HS alone (1C and 3C) and EBR+HS (1E and 3E) treatments as compared to the no-EBR and no-stress (0C) control. To focus solely on BR effects on gene expression, genes with ≥ 2-fold change by EBR under no-stress (0E *vs.* 0C; 273 genes), 1 h HS (1E *vs.* 1C; 239 genes) and 3 h HS (3E *vs.* 3C; 256 genes) conditions were identified by applying 2-way ANOVA to fold change analysis in Genespring. Collectively the 397 genes identified in this analysis have been termed as 'BR response genes'; this set of genes is expected to consist of both primary and secondary BR response genes. Comparison of changes at different time points revealed that 117 up-regulated and 23 down-regulated genes were common to the three time points (0, 1 and 3 h). It should be noted that some genes were changed at one or two time points only (Figure 4.6A). A mitochondrial transcription termination factor (mTERF)-related gene (At1g78930) showed a different trend than other genes; this gene was not just up-regulated at 1 h, but it was also down-regulated at 0 h and 3 h. Overall, these results indicate that BR effects extend across no-stress and stress conditions, and some changes are specific to certain time points.

#### *4.3.8b GO analysis of 'BR response genes'*

Gene networks affected by EBR at different time points were related to biological processes using the GO analysis tool available at TAIR (Figure 4.6B). Although majority of the genes belonged to unknown cellular processes, a high proportion (23% of all 'BR response genes') fell under the 'response to stress' category. Other categories were signal transduction, transcription, protein metabolism, development and cell organization/biogenesis. It is notable that genes correlated with DNA or RNA metabolism and electron transport/energy pathways were only down-regulated; there were no up-regulated genes in this category. About 10% of down-regulated genes belong

**Figure 4.4** Distribution of genes responsive to EBR, HS and EBR+HS into functional categories by MapMan. The normalized expression values of genes significantly changed under each condition were incorporated into MapMan pathway annotator. Genes significantly ($p < 0.05$) grouped into 10 functional bins were selected and arranged according to the functional term and gene expression at each treatment condition (0C, 0E, 1C, 3C, 1E and 3E). Each square represents a gene and the color of the square is indicative of the normalized expression level in response to corresponding treatment. To allow comparisons between functional categorization and expression patterns, number of genes from a functional bin that belong to a particular cluster (indicated by Cluster ID) are shown in front of the functional bin.

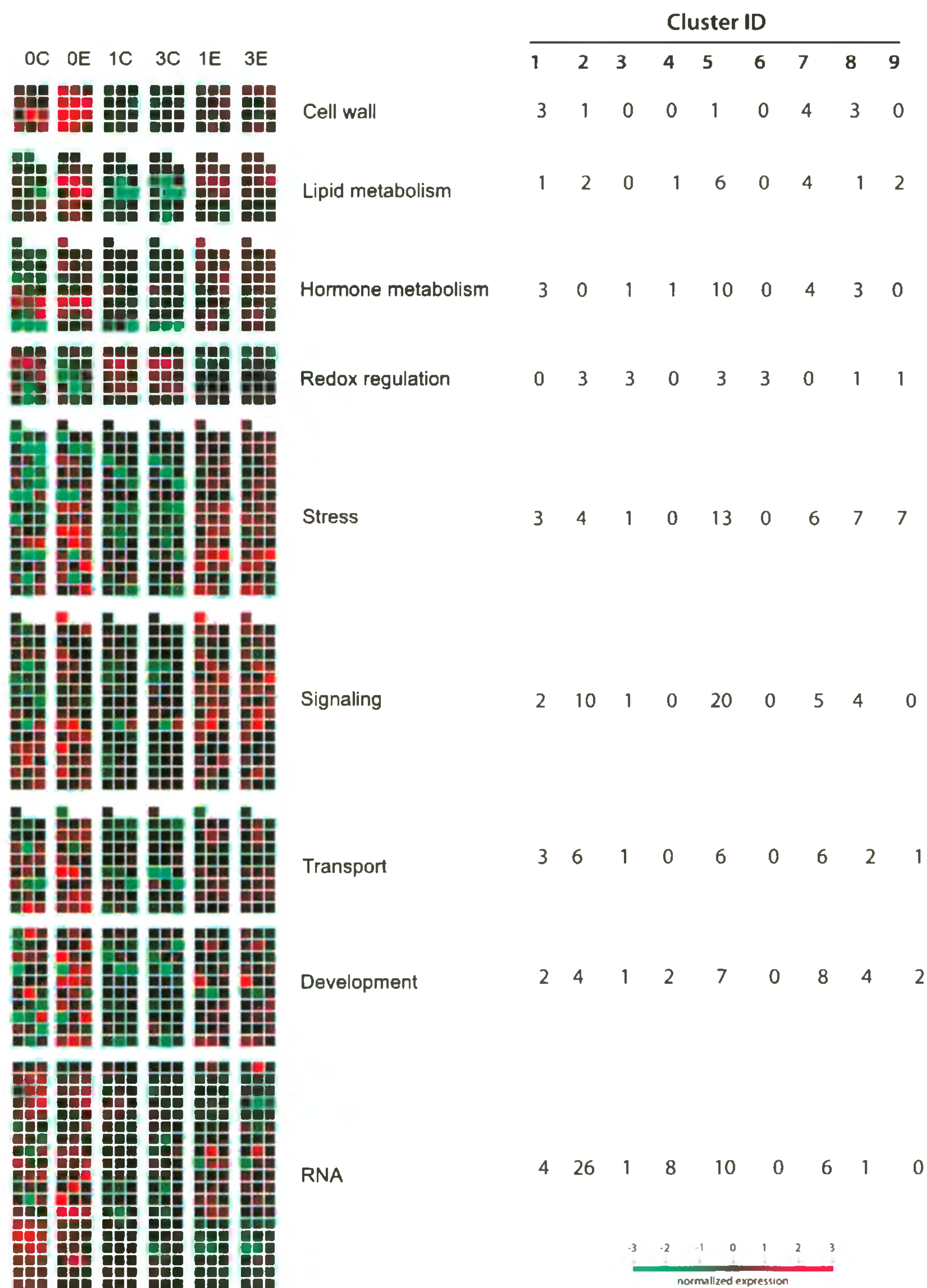
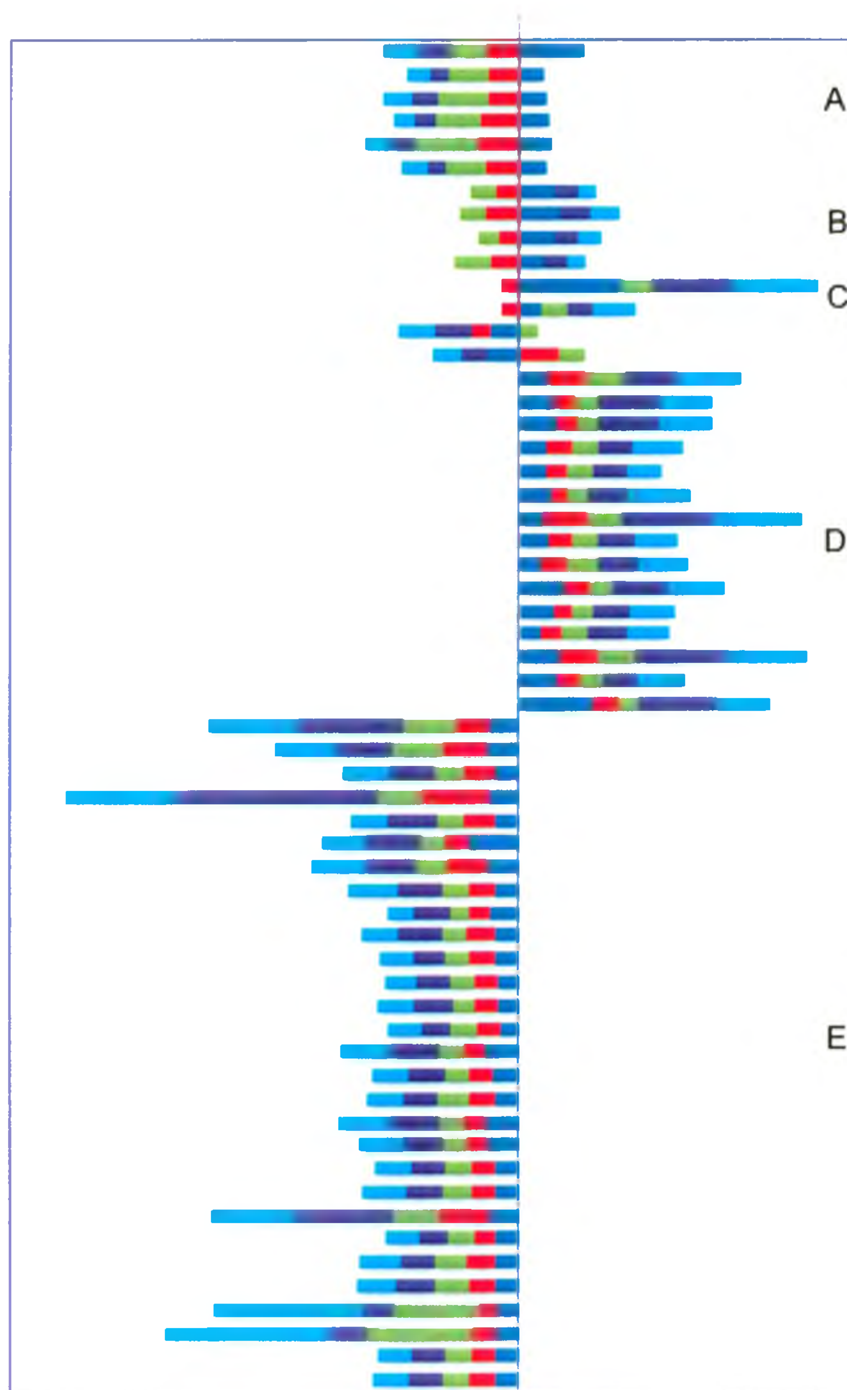

Cluster ID
0C 0E 1C 3C 1E 3E
1 2 3 4 5 6 7 8 9
Cell wall 3 1 0 0 1 0 4 3 0
Lipid metabolism 1 2 0 1 6 0 4 1 2
Hormone metabolism 3 0 1 1 10 0 4 3 0
Redox regulation 0 3 3 0 3 3 0 1 1
Stress 3 4 1 0 13 0 6 7 7
Signaling 2 10 1 0 20 0 5 4 0
Transport 3 6 1 0 6 0 6 2 1
Development 2 4 1 2 7 0 8 4 2
RNA 4 26 1 8 10 0 6 1 0
-3 -2 -1 0 1 2 3
normalized expression

**Figure 4.5** Grouping of protein metabolism genes based on expression patterns. A total of 58 genes enriched in protein category were identified by MapMan analysis of EBR-, HS- and EBR+HS-responsive genes. The genes were grouped (A to E) according to their expression patterns. The fold change (up- or down-regulation) in response to individual treatment (0E, 1C, 3C, 1E and 3E) relative to control (0C) is indicated for each gene.


A
B
C
D
E
down
up
Fold change
-10 -8 -6 -4 -2 0 2 4 6 8 10

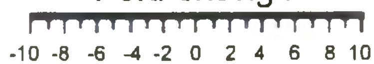

0E
1C
3C
1E
3E

**Figure 4.6** Overview of 'BR response genes'. A total of 397 genes responsive to EBR at 0 h (no-stress), 1 h and 3 h (HS) by $\geq 2$ -fold were identified by Genespring. These genes were subjected to venn diagram analysis and gene ontology (GO) annotation analysis. A) Venn diagram representing overlap between genes down-regulated by EBR at 0 h, 1 h and 3 h time points. B) Venn diagram representing overlap between genes up-regulated by EBR at 0 h, 1 h and 3 h time points. C) Functional categorization of 'BR responsive genes'. Putative functions of genes up- or down-regulated by EBR at 0 h, 1 h and 3 h were determined according to GO annotations from 'The Arabidopsis Information Resource' (TAIR) database. Number of genes in each functional category is indicated.

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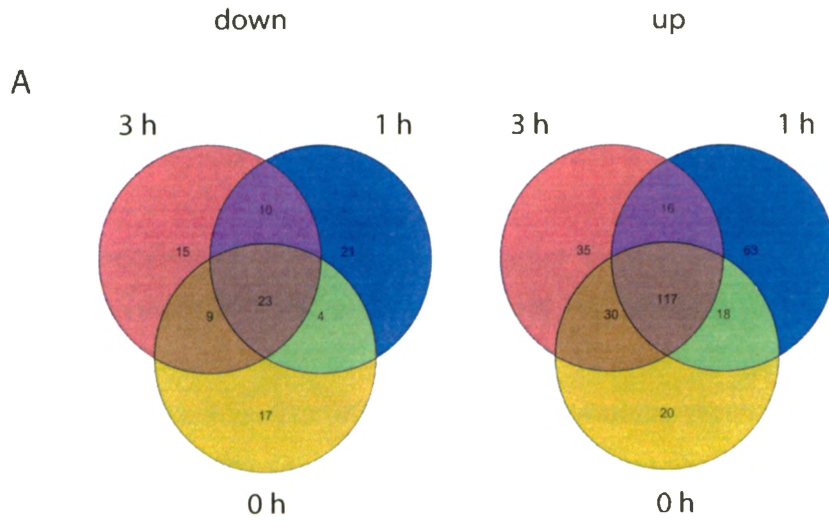
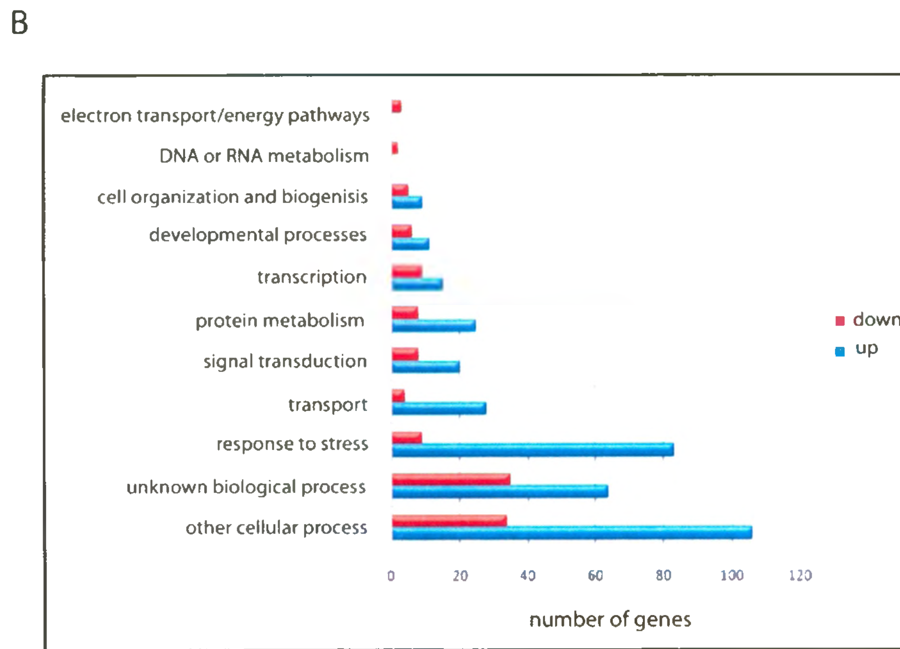

down
up
A
3 h
1 h
15
10
21
23
9
4
17
0 h
3 h
1 h
35
16
63
117
30
18
20
0 h


B
electron transport/energy pathways
DNA or RNA metabolism
cell organization and biogenisis
developmental processes
transcription
protein metabolism
signal transduction
transport
response to stress
unknown biological process
other cellular process
down
up
0
20
40
60
80
100
120
number of genes

to response to stress category, where as processes like transcription (9%), signal transduction (8%), protein metabolism (8%) and development (6%) were the other major categories. Among the up-regulated genes 27.8% belong to response to stress category followed by transport (9.4%) as the next major category of up-regulated genes. Thus, it would appear that up-regulated genes were predominantly related to stress, while the down-regulated genes were related to stress, development and regulatory processes.

### *4.3.8c Functional annotation of 'BR response genes' by MapMan*

The 'BR response genes' were incorporated into MapMan pathway annotator to obtain functional annotations. The Wilcoxon rank sum test assigned the genes to 9 MapMan bins (Table 4.3). The effect of EBR on various regulatory processes was analyzed by subjecting the set of genes to `regulation overview` pathway analysis in MapMan. Genes involved in processes like signaling, redox regulation, protein metabolism and hormone metabolism were found to be regulated by EBR (Figure 4.7). Each square in Figure 4.7 corresponds to a gene and the color of the square indicates fold-change at the lowest stress time point. The latter is explained as follows: to fulfill the requirement of the software for a single value, for a gene induced by EBR at 0 h, 1 h and 3 h, the value at 0 h was used as input.

### CELL SIGNALING CATEGORY

Out of the 397 genes changed by EBR, 54 (13.6%) belong to the signaling category; these encode 14 receptor kinases, one G-protein, one phospho-inositide, 5 calcium signaling proteins, 3 MAP kinases, and 30 transcription factors (Figure 4.7).

#### Receptor kinases

Of the 14 receptor kinases, 7 belong to the superfamily of LRR-RLKs (Figure 4.7). Four genes encoding LRR-RLKs were down-regulated, while two wall-associated kinases *(WAK1* and *WAK2)*, *ARK1 (A. THALIANA RECEPTOR KINASE 1)*, and *CRK10 (CYSTEINE-RICH RLK10)* were up-regulated by EBR. A link between BR and cell wall expansion is already known in published literature (Nakaya et al., 2002), however, it is interesting to note that *WAK1* and *WAK2* are SA-responsive and contain an EGF calcium-

binding domain. The *wak2* mutant plants exhibit a loss of cell expansion as well as dependence on sugars and salts for seedling growth (Kohorn et al., 2006). A role for WAK1 as a signaling receptor of extracellular matrix component has been reported (Decreux and Messiaen, 2005). ARK1 is involved in cytoskeletal organization to specify growth orientation in Arabidopsis root hairs (Yoo et al., 2008). CRK10 is shown to be responsive to pathogen elicitors (Benschop et al., 2007). Thus, the range of activities linked with different receptor kinases affected by BR includes development, defense and hormone responses.

**G-proteins and phospholipases**

The roles of G-proteins and phospholipases in various signaling process are well documented (Jones, 2002). Transcripts of *AtRABG2/AtRab7A (Arabidopsis Rab GTPase homolog G2)* and *ATPLC1 (PHOSPHOLIPASE C 1)* were induced by EBR. ATPLC1 is a phosphatidylinositol-specific phospholipase C that is strongly induced by environmental stresses, such as dehydration, salinity, and low temperature (Hirayama et al., 1995).

**Calcium signaling**

Calcium is important in the activation of biotic and abiotic stress-related signaling cascades (Kiegle et al., 2000). Five genes involved in calcium signaling that were induced by EBR include calmodulin-like calcium-binding protein (*CABP-22*, At2g41090), *RD20 (RESPONSIVE TO DESSICATION 20)*, *EDA39 (EMBRYO SAC DEVELOPMENT ARREST 39)* and two calmodulin-binding proteins (At4g25800 and At5g57580). The expression of *RD20* gene was known to be induced by drought, ABA and high salinity (Takahashi, 2000).

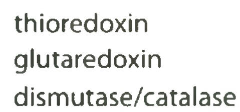
**MAP kinases**

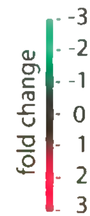
Several MAP kinases are preferentially activated by environmental stresses and are actively involved in various stress responses (Nakagami et al., 2005). MAP kinases that were significantly altered by BR at the transcript level include *MPK3 (MITOGEN-ACTIVATED PROTEIN KINASE 3)*, *MPK19 (MITOGEN-ACTIVATED PROTEIN KINASE 19)* and *MEK1 (MAP KINASE KINASE 1)*. *MPK3* transcript levels increase in

**Table 4.3** MapMan functional bins enriched with genes significantly changed by EBR, HS and EBR+HS.

| | MapMan bin | No. of genes | *p*-value |
|---|---|---|---|
| 1 | redox regulation | 10 | 4.421E-5 |
| 2 | miscellaneous | 41 | 7.104E-5 |
| 3 | Stress | 30 | 0.004 |
| 4 | RNA | 34 | 0.031 |
| 5 | secondary metabolism | 15 | 0.036 |
| 6 | not assigned | 111 | 0.038 |
| 7 | lipid metabolism | 8 | 0.038 |
| 8 | Protein | 26 | 0.051 |
| 9 | DNA | 3 | 0.099 |

**Figure 4.7** Functional categorization of 'BR response genes' by MapMan. Genes responsive to EBR by $\geq$ 2-fold at 0 h (no-stress), 1 h and 3 h (HS) time points were grouped into various functional categories by `regulation overview` analysis in MapMan. Number of BR response genes is indicated for each functional category. Each square corresponds to a gene and the color of the square indicates fold change at the lowest stress time point

| functional category | gene expression |
|---|---|
| **Signaling** | |
| receptor kinases | |
| G-proteins | |
| phospho-inositides | |
| calcium signaling | |
| MAP kinases | |
| transcription factors | |
| **Redox** | |
| thioredoxin |  |
| glutaredoxin | |
| dismutase/catalase | |
| **Protein** | |
| modification | |
| degradation | |
| **Hormone** | |
| IAA | |
| ABA | |
| BR | |
| ET | |
| JA | |
| SA | |
| GA | |
| cytokinin | |



response to touch, cold, salinity and chitin oligomers (Mizoguchi et al., 1996; Ramonell et al., 2005). MEK1 functions in a stress-activated MAPK pathway by phosphorylating MPK4 (MITOGEN-ACTIVATED PROTEIN KINASE 4) in response to stress and gets phosphorylated by MEKK1 (MAP KINASE KINASE KINASE 1) in response to wounding (Gao et al., 2008).

**Transcription factors**

Transcription factors constitute a large proportion of the signaling genes (56%) affected by BR, of which 15 were up-regulated and 15 were down-regulated. The down-regulated genes include members of the Arabidopsis response regulator (ARR) family *(ARR7, ARR 15, ARR 4* and *ARR16)*. ARRs are involved in cytokinin and development responses (To et al., 2007). Down-regulation of ARRs by BR has been noted in earlier studies (Vert et al., 2005). Three CCAAT-binding transcription factors were up-regulated. Two members of the WRKY gene family, *WRKY70* and *WRKY33,* were up-regulated by EBR, while *WRKY17* was down-regulated. These WRKY genes have key roles in defense and abiotic stress reponses (Eulgem et al., 2000). Transcription factors belonging to MYB, MADS box and zinc finger families were also changed in expression by EBR.

## REDOX REGULATION

Six genes belonging to the glutaredoxin family and 2 dismutases *(CCS1, COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE 1* and *CSD2, COPPER/ZINC SUPEROXIDE DISMUTASE 2)* were down-regulated. One thioredoxin (At4g26160) and a catalase, *CAT3 (CATALASE 3)* that catalyzes the breakdown of hydrogen peroxide ($H_2O_2$) into water and oxygen (Du et al., 2008), were up-regulated by EBR.

## PROTEIN MODIFICATION

Several proteins are activated or inactivated by post-translational modifications. Genes involved in post-translational modifications include kinases, phosphatases, methylases and other enzymes. Transcripts of 6 protein kinases that were induced by EBR include *MAPKKK10 (MITOGEN-ACTIVATED PROTEIN KINASE KINASE*

*KINASE 10)*, a shaggy-related protein kinase beta / ASK-beta *(ASK2)*, *APK2A (PROTEIN KINASE 2A)*, and 3 protein kinase family proteins (At4g17660, At2g28590, At5g47750). Transcripts of an S-adenosyl-L-methionine:carboxyl methyltransferase family protein (At3g44860) and protein phosphatase 2C (At3g51370) were up-regulated, while another protein phosphatase 2C (At3g51370) was down-regulated.

**PROTEIN DEGRADATION**

Majority of the up-regulated genes within this functional category belong to the zinc finger (C3HC4-type RING finger) family. Two serine carboxypeptidases (*SCPL35*; At5g08260 and *SCPL51*; At2g27920), an AAA-type ATPase family gene (At4g28000), an aspartyl protease family gene (At1g62290), a subtilase family gene (At5g59120) and a cysteine protease inhibitor (At4g16500) were up-regulated by EBR, and *UBC17 (UBIQUITIN-CONJUGATING ENZYME 17)* and *ATL8* (At1g76410) were down-regulated.

**PHYTOHORMONES**

Several genes involved in hormone responses were significantly changed in expression by EBR (Figure 4.7), including a BR-related gene, *BAS1 (PHYB ACTIVATION TAGGED SUPPRESSOR 1)*. *BAS1* encodes a member of the cytochrome p450 family that serves as a control point between multiple photoreceptor systems and BR signal transduction (Neff et al., 1999). Three ABA-related genes, including *KIN1* (At5g15960), were up-regulated, while one ABA-related gene was down-regulated. *KIN1* is a well-established ABA-, cold-, dehydration- and mannitol- responsive gene (Wang et al., 1995). Two genes involved in JA biosynthesis, *LOX2 (LIPOXYGENASE 2)* and *OPR3 (12-OXOPHYTODIENOATE REDUCTASE)*, and 2 auxin-related genes *NIT2 (NITRILASE 2)* and At4g36110 were up-regulated, while one auxin-responsive gene (At3g25290) was down-regulated. *NIT2* encodes a nitrilase, which catalyzes the hydrolysis of indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA) (Bartel and Fink, 1994). An ethylene (ET)-related transcription factor, *AtERF15 (ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 15)*, and salicylic acid (SA)-related methyl transferase (At3g44870) and transcription factor *WRKY70*, were up-regulated by

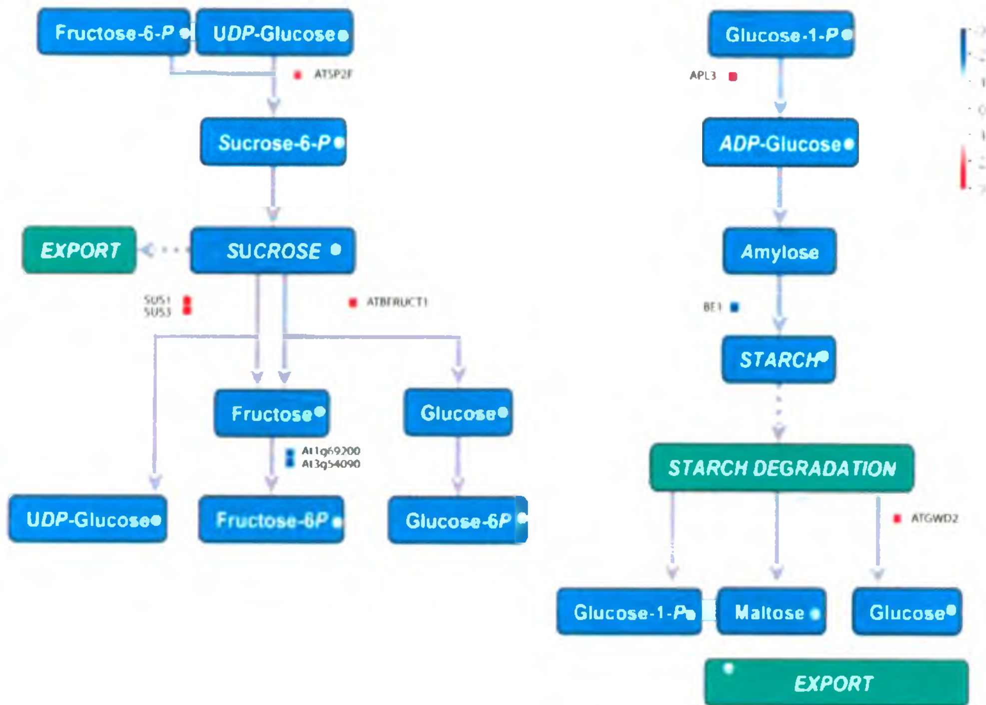
BR. It is to be noted that WRKY70 is a central regulator of SA and JA/ET pathways (Li et al., 2004). The induction of *WRKY70* by BR in SA mutants and in short-term BR treatment has been described in chapter 3 of this thesis. These results together make a strong case for *WRKY70* as a primary response gene of BR. Cytokinin- and gibberellic acid (GA)-related transcripts were also regulated by EBR. The expression of *UGT76C2 (UDP-GLUCOSYL TRANSFERASE 76C2),* a cytokinin-N-glucosyltransferase, was down-regulated, while a GA-related gene (At2g14900) was up-regulated by EBR. BR effect on key genes involved in other hormone pathways is indicative of its interaction with these hormones in mediating diverse developmental and stress responses.

***4.3.8d Effect of EBR on sucrose-starch metabolism***

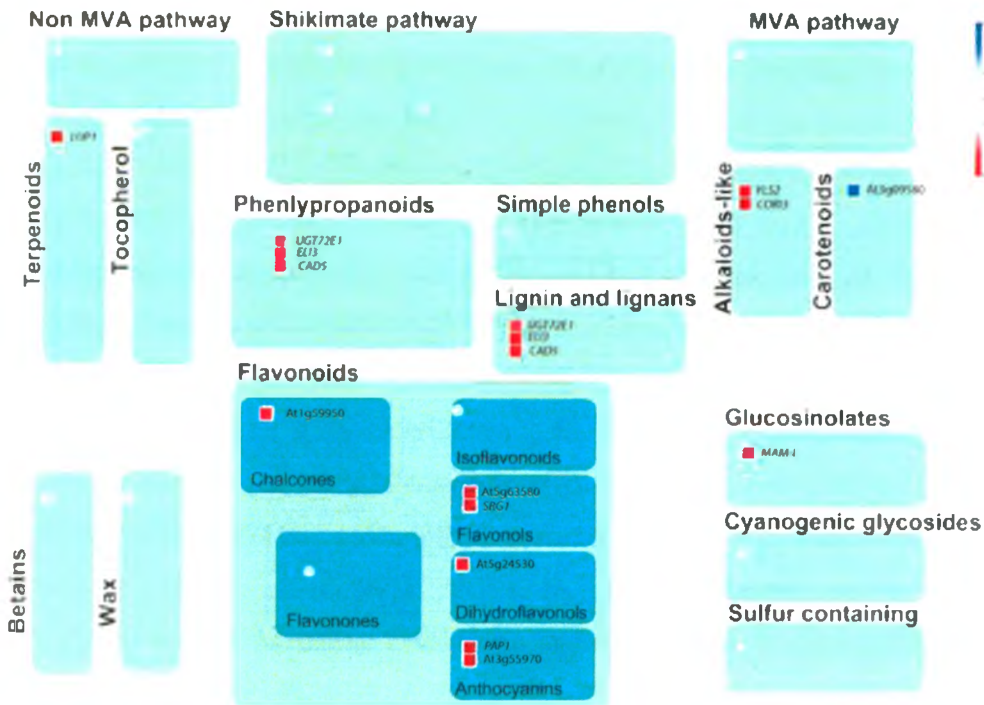
MapMan analysis of the 'BR response genes' revealed that several sucrose and starch metabolism genes were affected by EBR (Figure 4.8A). Six sucrose-related genes were changed; 4 were up-regulated and 2 genes were down-regulated (Figure 4.8A). Of the up-regulated genes, *SUS1 (SUCROSE SYNTHASE 1), SUS3 (SUCROSE SYNTHASE 3)* and *AtBFRUCT1/CWINV1 (CELL WALL INVERTASE 1)* are involved in sucrose degradation, while *SPS2F (SUCROSE PHOSPHATE SYNTHASE 2F)* is involved in sucrose synthesis. The down-regulated genes include two fructokinases (At1g69200 and At3g54090) involved in the conversion of Fructose to Fructose-6-phosphate (Figure 4.8A). Three starch metabolism genes were identified; an alpha-amylase *(BE1; BRANCHING ENZYME 1)* involved in the conversion of amylase to starch was down-regulated, while *APL3* encoding the large subunit of ADP-Glucose Pyrophosphorylase that catalyzes the first, rate-limiting step in starch biosynthesis (Villand et al., 1992), and *AtGWD2/ PWD (PHOSPHOGLUCAN, WATER DIKINASE)* that promotes starch degradation, were up-regulated (Figure 4.8A). It is tentatively concluded from these results that BR promotes formation of simple, soluble sugars like fructose and glucose during stress conditions.

**Figure 4.8** MapMan overview of 'BR response genes' involved in carbohydrate and secondary metabolism. **A)** Schematic representation of 'BR response genes' involved in sucrose-starch metabolism. The gene names or AGI identifiers of genes involved at various steps of the pathway are indicated. Each square corresponds to a gene and the color of the square indicates fold change at the lowest stress time point. **B)** Schematic representation of 'BR response genes' involved in secondary metabolism. The gene names or AGI identifiers of genes involved at various steps of the pathway are indicated. Each square corresponds to a gene and the color of the square indicates fold change at the lowest stress time point.

A




B



#### *4.3.8e Effect of EBR on secondary metabolism*

An overview of secondary metabolism was examined in MapMan to determine which categories were significantly changed by EBR treatment. Fifteen genes involved in different secondary metabolism pathways were identified (Table 4.3, Figure 4.8B).

**Flavonoid metabolism**

Six genes affecting the metabolism of various groups of flavonoids were up-regulated by EBR (Figure 4.8B). An aldo/keto reducatase (At1g59960) and an oxidoreductase (At5g24530) involved in chalcone and dihydroflavonoid metabolism, respectively, and 2 genes each involved in flavonol metabolism (*SRG1*, At1g17020; flavonol synthase, At5g63580) and anthocyanin (*PAP1*, At1g56650; oxidoreductase, At3g55970) were up-regulated by EBR. The importance of flavonoids as antioxidants and plant protectants against pathogens and abiotic stresses is well established (Pourcel et al., 2007). These results support a role for BR in influencing flavonoid levels, which in turn may impact stress tolerance.

**Lignin metabolism**

Lignins are synthesized by a branch on the phenylproponoid pathway (Hahlbrook and Scheel, 1989), and cinnamyl alcohol dehydrogenases (CADs) are enzymes responsible for lignin biosynthesis (Walter et al., 1988). Transcripts of 2 *CADs*, *CAD5 (CINNAMYL ALCOHOL DEHYDROGENASE 5)* and the pathogen defense-related *ELI3 (ELICITOR-ACTIVATED GENE 3),* were up-regulated by EBR (Figure 4.8B). Another group of enzymes involved in the phenylproponoid pathway are glucosyl transfereases (GTs). The expression of *UGT72E1 (UDP-glucosyl transferase 72E1),* was also up-regulated by EBR.

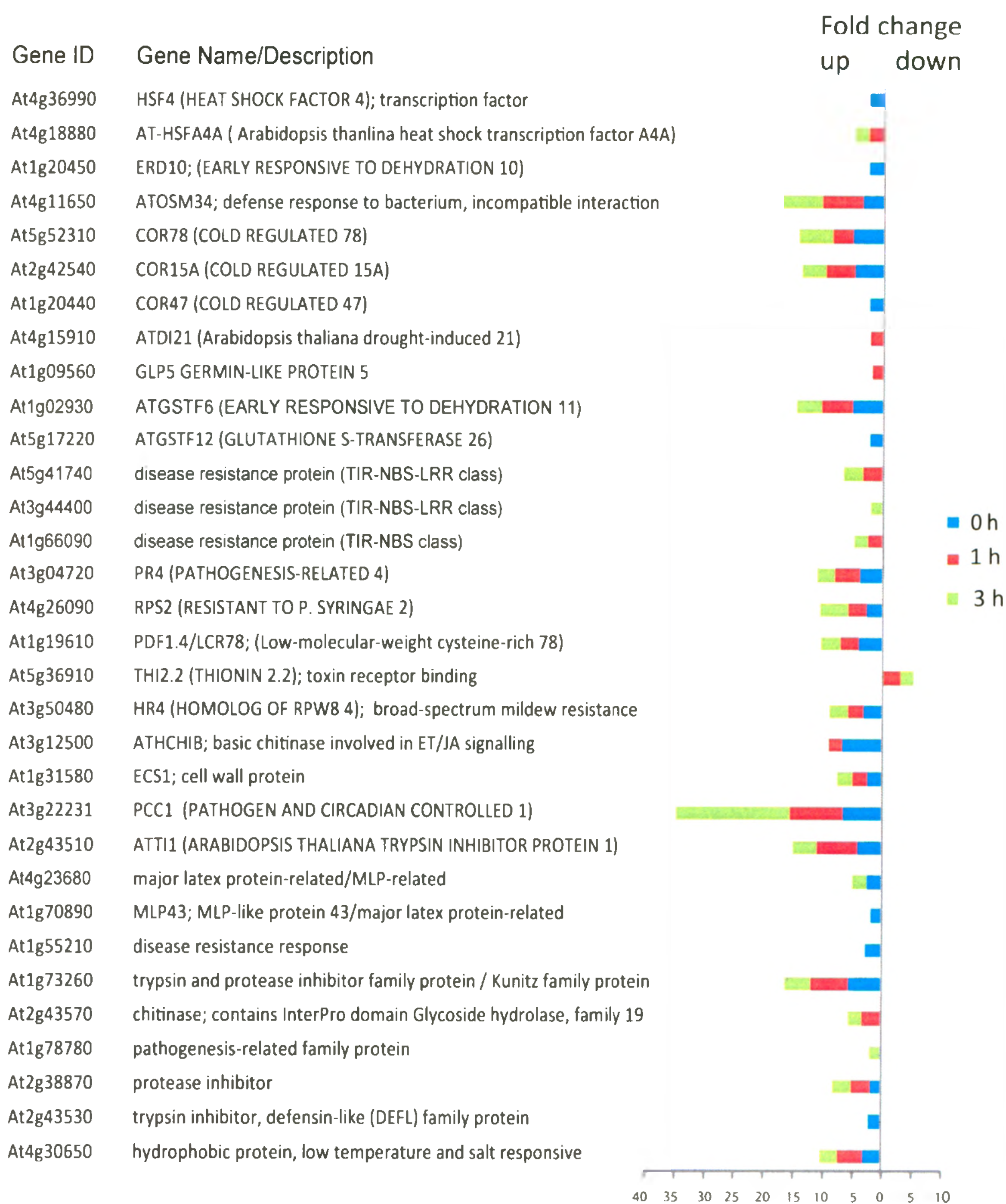
Several other secondary metabolism genes, such as *YLS2* (At3g51430) and *CORI3* (At4g23600) involved in alkaloid metabolism, *LUP1* (At1g78970) related to terpenoid metabolism, *MAM-L* (At5g23020) related to glucosinolate metabolism, and an amino oxidase family gene (At3g09580) related to carotenoid metabolism, were changed by EBR. Thus, several secondary metabolism pathways appear to be affected by BR, which are likely to contribute to BR-mediated stress tolerance.

***4.3.8f AtGenExpress Visualization Tool (AVT) analysis of 'BR response genes'***

According to GO analysis a high percentage of the 'BR response genes' were stress-related. These genes were pinpointed using MapMan annotator. A total of 30 genes were significantly enriched in the stress bin of MapMan (Table 4.3). The fold-change values of these genes in response to EBR at 0 h, 1 h and 3 h time points are shown in Figure 4.9. Genes *AtGSTF6* (At1g02930) and *AtGSTF12* (At5g17220) were originally listed in the miscellaneous category of MapMan, but are included here due to their known roles in oxidative stress (Roxas et al., 2000). Out of these 32 genes, 14 were changed by EBR at all time points, whereas 5 were changed at 1 h and 3 h of HS (Figure 4.9). It is of interest that 7 stress-related genes, including heat shock transcription factor *HSF4* (At4g36990), *ERD10* (At1g20450) and pathogen defense-related genes, were up-regulated by EBR under no-stress (0 h) conditions, but not at 1 and 3 h of HS. The up-regulation of another heat shock transcription factor *AtHSFA4A* (At4g18880) at 1 h and 3 h of HS suggests a role for BR in controlling heat stress responses. Thus, in summary, BR affects the expression of stress-related genes under both no-stress and stress conditions.

A large-scale genome wide expression profiling data in response to various environmental and chemical cues under well-defined and reproducible conditions has been generated for Arabidopsis (AtGenExpress; http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp). Web interactive visualization of this data is made possible through AtGenExpress Visualization Tool (AVT; http://jsp.weigelworld.org/expviz/expviz.jsp). The EBR-response genes were searched for response to abiotic stresses by AVT (Kilian et al., 2007). The mean normalized expression intensity of each gene in response to different stress treatments is shown in Figure 4.10A. Each colored box represents the expression intensity of a gene in response to the corresponding stress treatment. Several of the genes in question were seen to be responsive to drought, salt, osmotic, wounding and UV-B stresses (Figure 4.10A). This information reinforces the role of BR in directly or indirectly influencing the expression of a wide range of stress-regulated genes.

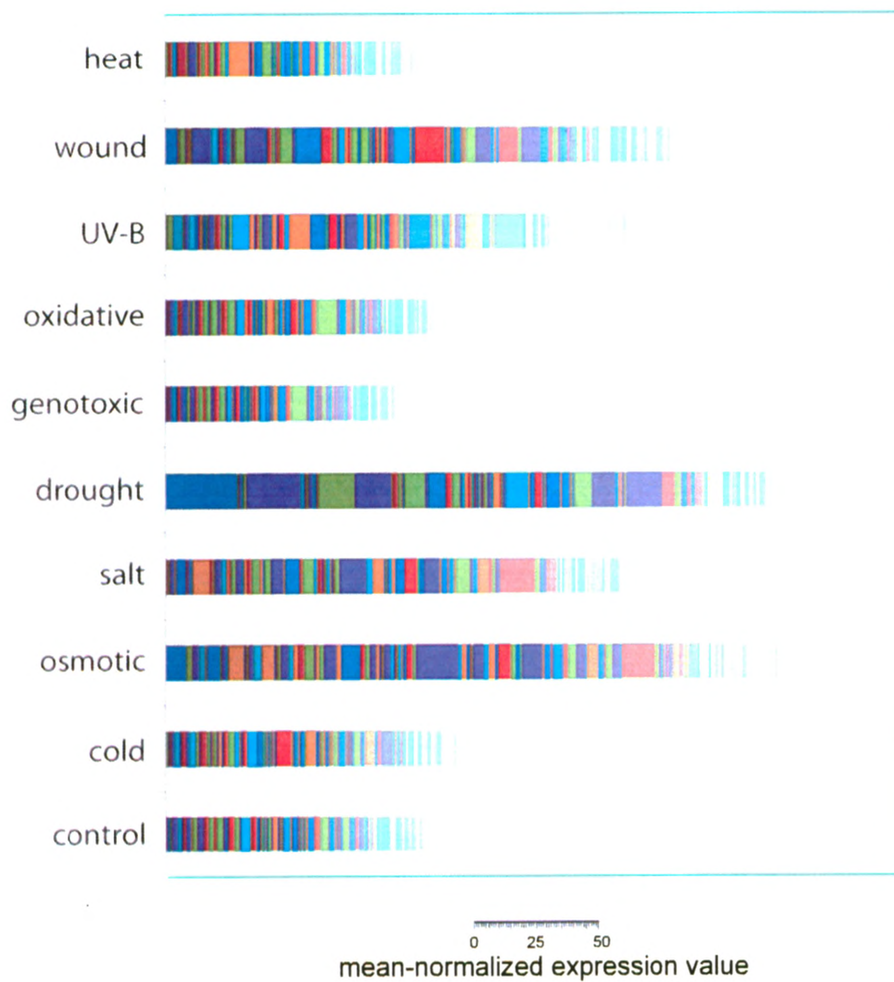
**Figure 4.9** Overview of 'BR response genes' with stress-related roles. Genes with stress-related roles were identified by MapMan analysis. The fold change values (up- or down-regulation) of these genes in response to EBR at 0 h (no-stress), 1 h and 3 h (HS) time points are indicated along with their AGI identifier and gene name or description.



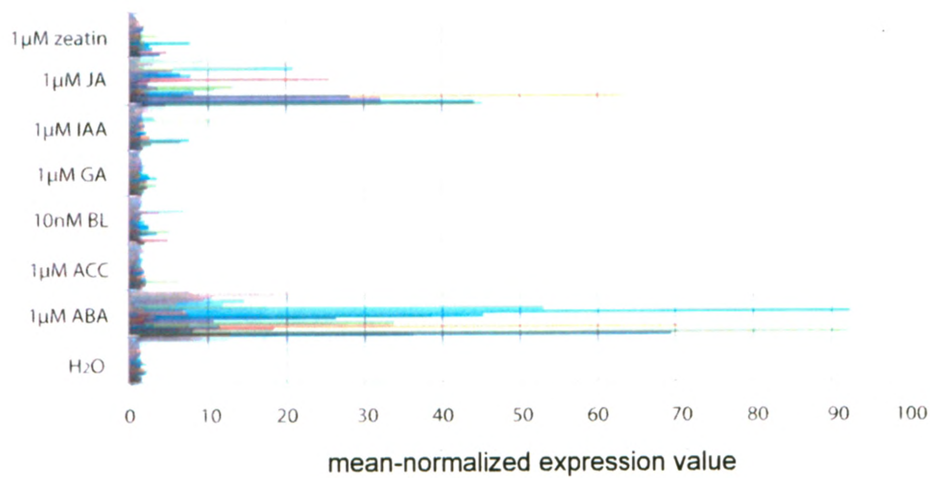
Gene ID
Gene Name/Description
Fold change
up down
At4g36990 HSF4 (HEAT SHOCK FACTOR 4); transcription factor
At4g18880 AT-HSFA4A ( Arabidopsis thanlina heat shock transcription factor A4A)
At1g20450 ERD10; (EARLY RESPONSIVE TO DEHYDRATION 10)
At4g11650 ATOSM34; defense response to bacterium, incompatible interaction
At5g52310 COR78 (COLD REGULATED 78)
At2g42540 COR15A (COLD REGULATED 15A)
At1g20440 COR47 (COLD REGULATED 47)
At4g15910 ATDI21 (Arabidopsis thaliana drought-induced 21)
At1g09560 GLP5 GERMIN-LIKE PROTEIN 5
At1g02930 ATGSTF6 (EARLY RESPONSIVE TO DEHYDRATION 11)
At5g17220 ATGSTF12 (GLUTATHIONE S-TRANSFERASE 26)
At5g41740 disease resistance protein (TIR-NBS-LRR class)
At3g44400 disease resistance protein (TIR-NBS-LRR class)
At1g66090 disease resistance protein (TIR-NBS class)
At3g04720 PR4 (PATHOGENESIS-RELATED 4)
At4g26090 RPS2 (RESISTANT TO P. SYRINGAE 2)
At1g19610 PDF1.4/LCR78; (Low-molecular-weight cysteine-rich 78)
At5g36910 THI2.2 (THIONIN 2.2); toxin receptor binding
At3g50480 HR4 (HOMOLOG OF RPW8 4); broad-spectrum mildew resistance
At3g12500 ATHCHIB; basic chitinase involved in ET/JA signalling
At1g31580 ECS1; cell wall protein
At3g22231 PCC1 (PATHOGEN AND CIRCADIAN CONTROLLED 1)
At2g43510 ATTI1 (ARABIDOPSIS THALIANA TRYPSIN INHIBITOR PROTEIN 1)
At4g23680 major latex protein-related/MLP-related
At1g70890 MLP43; MLP-like protein 43/major latex protein-related
At1g55210 disease resistance response
At1g73260 trypsin and protease inhibitor family protein / Kunitz family protein
At2g43570 chitinase; contains InterPro domain Glycoside hydrolase, family 19
At1g78780 pathogenesis-related family protein
At2g38870 protease inhibitor
At2g43530 trypsin inhibitor, defensin-like (DEFL) family protein
At4g30650 hydrophobic protein, low temperature and salt responsive
0 h
1 h
3 h
40 35 30 25 20 15 10 5 0 5 10

**Figure 4.10** *In silico* expression analysis using AtGenExpress Visualization Tool (AVT; http://jsp.weigelworld.org/expviz/expviz.jsp). The expression patterns of 'BR response genes' and BR*only* genes in response to abiotic stress and hormone treatments, respectively, were generated using AVT. **A)** The normalized expression values of 'BR response genes' in response to a 3 h exposure to different abiotic stresses are shown. The stress conditions were cold, 3 h at 4°C; osmotic, 300 mM mannitol for 3 h; salt, 150 mM NaCl for 3 h; drought, 15 min dry air stream followed by incubation in climate chamber for 3 h; oxidative, 10 M methyl viologen for 3 h; UV light, 0.25 h UV-B light field for 3 h; wounding, leaf punctuation and tissues collected after 3 h; heat, 3 h at 38°C. **B)** The normalized expression values of BR*only* genes in response to a 3 h treatment with different hormones.

A




B



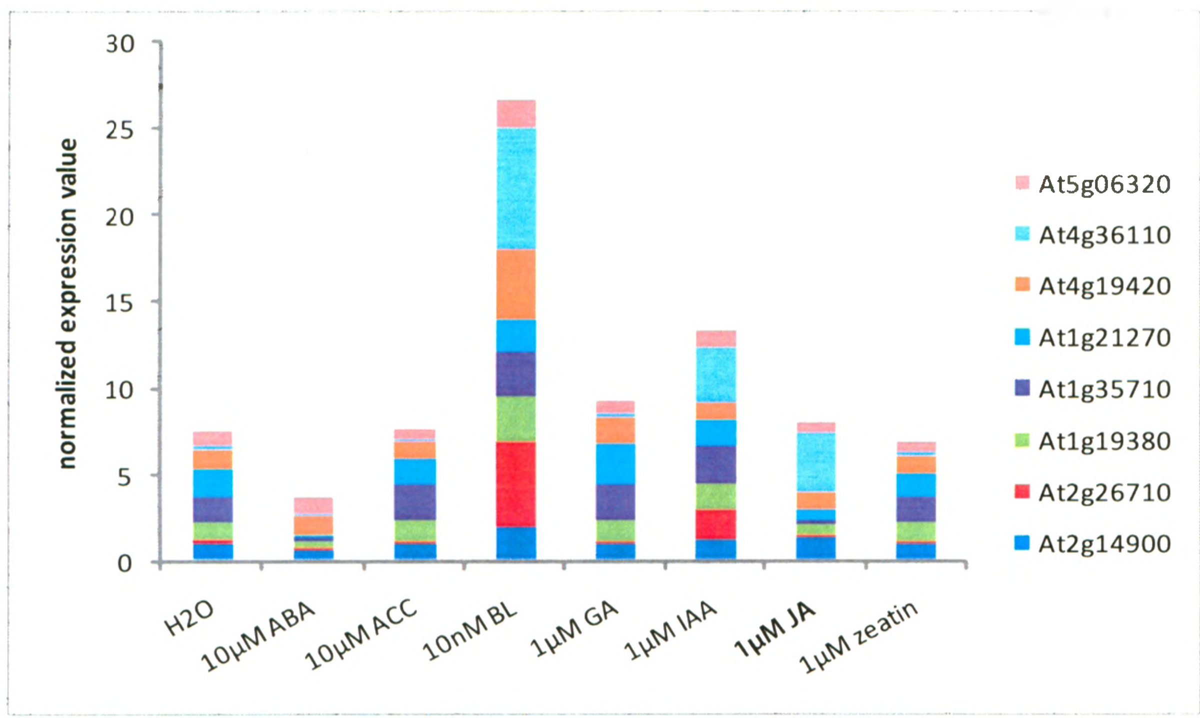
To confirm MapMan annotation of hormone-related genes, BR response genes at no-stress condition (0E *vs.* 0C) were analyzed for response to different plant hormones using AVT. These genes are termed as BR*only* (0 h only) to distinguish them from 'BR response genes', which include all time points. Analysis of the 273 BR*only* genes revealed that there is predominant regulation by ABA followed by JA in terms of both gene numbers and magnitude of change (Figure 4.10B). Treatment with 1-aminocyclopropane-1-carboxylic acid (ACC, an ET precursor), GA, IAA, and zeatin also impact expression of genes in this subset, but at a much lower level than ABA. AVT, GO and regulatory motif analyses all confirmed ABA-responsiveness of several genes, lending support to the idea that BR induces ABA levels, which in turn impacts expression of these genes. While BR may also share gene targets with ABA, the high expression values associated with these genes in our data suggests that the changes were impacted by ABA. Possible increase in JA levels by BR can also be used to explain the BR-JA relationship evident in our data, though overlapping responses of BR with JA and other hormones is an equally strong possibility.

***4.3.8g BRonly genes maximally up-regulated by BR in the AVT dataset***

Of the 273 BR*only* genes, 8 genes were found to be maximally induced by BR as compared to other plant hormones. The normalized expression intensity of the 8 genes in response to different hormone treatments is shown in Figure 4.11A. Their expression pattern in response to EBR under no-stress (0 h) and heat stress (1 h and 3 h) is shown in Figure 4.11B. The previously known BR-responsive gene, *BAS1* (Neff et al., 1999), an auxin-responsive gene (At4g36110), and a pectinacetylesterase family gene (At4g19420), were induced by EBR only under no-stress conditions, *WAK2* (At1g21270) and At1g35710 were induced under no-stress and 1 h HS conditions, while At5g06320, At1g19380 and At2g14900 were induced by EBR at all time points. GO analysis indicated involvement of these genes in diverse cellular processes such as defence, hormone and light responses, cellular homeostasis and signaling (Table 4.4). It is interesting to note that with the exception of At4g19420, the other 7 genes were down-regulated by ABA (Figure 4.11A). Thus, the relationship of BR with ABA is both of cooperation and antagonism.

**Figure 4.11** Expression profiles of BR maximally up-regulated genes. **A)** The normalized expression values of BR maximally up-regulated genes in response to a 3 h treatment with different hormones generated by AVT. **B)** The fold change in expression of BR maximally up-regulated genes in response to EBR at 0 h (no-stress), 1 h and 3 h (HS) time points.

A

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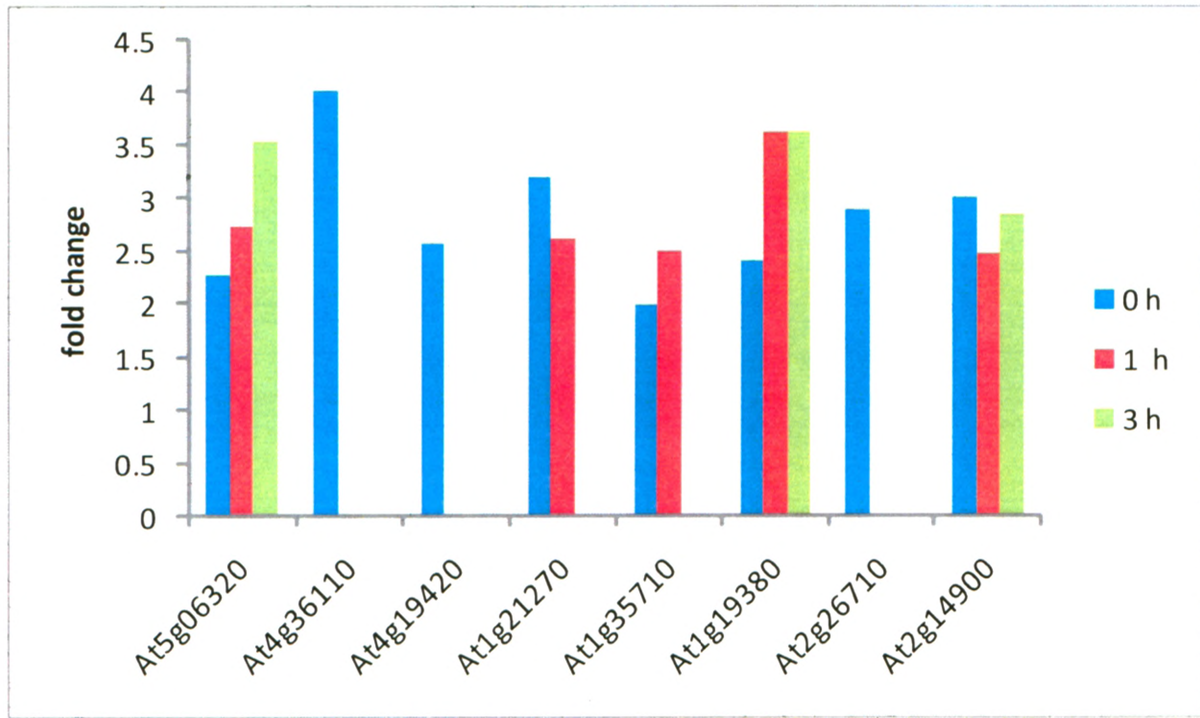


**Table 4.4** GO annotations (biological process) of BR maximally up-regulated genes.

| Gene ID | Gene name/ general information | GO process |
|---|---|---|
| At5g06320 | NHL3 (NDR1/HIN1-like 3) | defense response to bacterium |
| At4g36110 | auxin-responsive protein | response to auxin stimulus |
| At4g19420 | pectinacetylesterase family protein | carboxylesterase activity |
| At1g21270 | WAK2 (WALL ASSOCIATED KINASE 2) | cellular water homeostasis |
| | | oligosaccharide metabolic process |
| | | response to salicylic acid stimulus |
| At1g35710 | protein coding | transmembrane receptor protein tyrosine kinase signaling pathway |
| At1g19380 | protein coding | biological_process_unknown |
| At2g26710 | BAS1/CYP734A1 | response to brassinosteroid stimulus |
| | (PHYB ACTIVATION TAGGED SUPPRESSOR 1) | response to light stimulus |
| | | oxygen binding |
| At2g14900 | gibberellin-regulated family protein | response to gibberellin stimulus |

### *4.3.9 Functional validation of Microarray data*

### *4.3.9a Selection of candidate genes*

To see if new and previously characterized, but with no evidence of a role in stress, genes uncovered as 'BR response genes' in our microarray study have stress-related functions, four such genes (Table 4.5) were analyzed using T-DNA insertion mutants. The selected genes were two transcription factors (*WRKY33* and *WRKY17*), an acid phosphatase (*ACP5*) and a previously uncharacterized *LRR-RLK* (At1g35710), hereafter called as *BR RESPONSIVE RECEPTOR LIKE KINASE (BRRLK)*. It is notable that *BRRLK* is one of 8 genes that are maximally up-regulated by BR (Figure 4.11A) and has no known functions. WRKY33 and WRKY17 are known for their roles in pathogen defense. WRKY33 is a positive regulator of defense responses (Zheng et al., 2006), while WRKY17 acts as a negative regulator of basal resistance to *Pseudomonas syringae* pv. *tomato* (Journot-Catalino et al., 2006). ACP5 is involved in phosphate mobilization and in the metabolism of reactive oxygen species (del Pozo et al., 1999). However, the abiotic stress-related roles for these genes are not known. In order to form an initial idea of the structure – function relationships of the encoded products, the amino acid sequences were analyzed using the NCBI conserved domain database. The protein structure maps were generated using PROSITE-MyDomains-Image Creater tool (http://ca.expasy.org/cgi-bin/prosite/mydomains/). As shown in Figure 4.12, WRKY17 consists of a WRKY domain and a calmodulin binding domain (CaMBD), whereas WRKY33 has two WRKY domains. The WRKY domain containing proteins act as transcription factors by binding to the W-box promoter motif of the target genes (Eulgem et al., 2000). ACP5 consists of a protein phosphatase 2C (PP2C) domain involved in protein dephoshorylation. BRRLK consists of a LRR (leucine-rich repeat) domain; a LRR_RI (LRRs, ribonuclease inhibitor [RI]) domain; and a protein serine/threonine kinase (S_TK) domain. These structural features indicate a receptor like kinase activity for BRRLK.

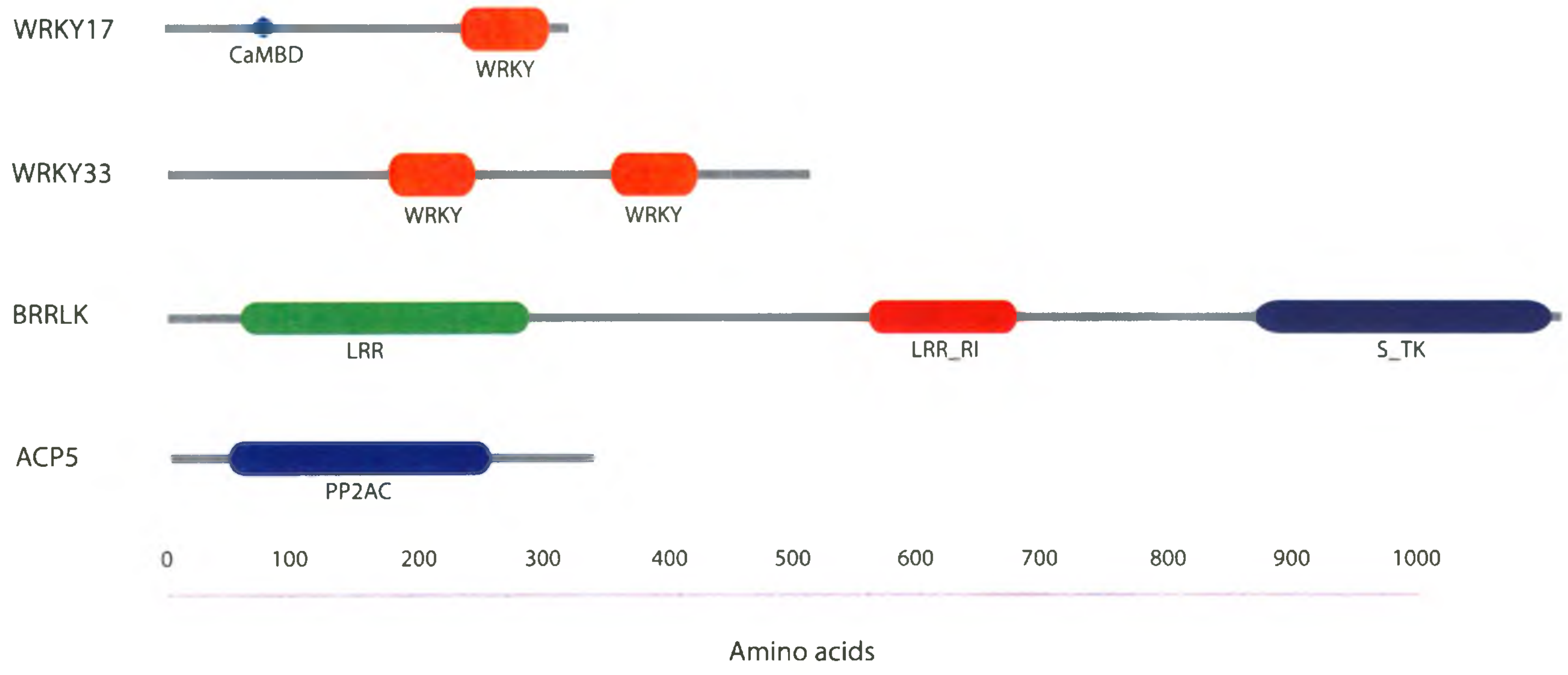
### *4.3.9b AVT analysis of candidate genes*

In our study, *WRKY17* and *WRKY33* were responsive to EBR only during HS. *WRKY17* was down-regulated by EBR at 1 h of HS; whereas *WRKY33* was up-regulated at 1 h and 3 h of HS by EBR. *ACP5* and *BRRLK* were up-regulated at both no-stress (0 h)

**Table 4.5** Genes selected for functional validation of Microarray data.

| AGI Code | Gene Name | Description | function | biological process | component |
|---|---|---|---|---|---|
| At2g24570 | ***WRKY17*** | WRKY17 (WRKY DNA-binding protein 17); transcription factor | calmodulin binding, transcription factor activity | defense response to bacterium | nucleus |
| At2g38470 | ***WRKY33*** | WRKY33 (WRKY DNA-binding protein 33); transcription factor | transcription factor activity | response to biotic and abiotic stress | nucleus |
| At1g35710 | ***BRRLK*** | leucine-rich repeat transmembrane protein kinase, putative | ATP binding, protein kinase activity | transmembrane receptor kinase signaling pathway, protein amino acid phosphorylation | endomembrane system |
| At3g17790 | ***ACP5*** | ATACP5 (acid phosphatase 5) | acid phosphatase activity | phosphate ion homeostasis, response to hydrogen peroxide | cell surface |

**Figure 4.12** Protein structures of the selected candidate genes indicating putative functional domains. Amino acid sequences were analyzed using the National Center for Biotechnology Information (NCBI) conserved domain database. The protein structure maps were generated using PROSITE-MyDomains-Image Creater tool (http://www.expasy.ch/tools/mydomains/).



WRKY17
CaMBD
WRKY
WRKY33
WRKY
WRKY
BRRLK
LRR
LRR_RI
S_TK
ACP5
PP2AC
0
100
200
300
400
500
600
700
800
900
1000
Amino acids

and HS (1 h and 3 h) time points (Figure 4.13A). To see how the selected genes respond to short-term treatment with BR, their expression in response to hormones was analyzed using AVT. The mean normalized expression values in response to a time course (0.5 h, 1 h and 3 h) treatment with various hormones are shown in Figure 4.13B. In line with our data, *WRKY17* was down-regulated by BR, but at all time points (Figure 4.13B). A slight up-regulation of *WRKY33* by BR occurred at 3 h, but a strong response to ABA and IAA occurred at 0.5 h (Figure 4.13B). In our data, *WRKY33* was up-regulated by EBR only during HS; thus it was a stress response heightened by BR. *ACP5* had slight up-regulation by BR at 0.5 h and 1 h, but was highly up-regulated by ABA at 3 h. *BRRLK* was maximally up-regulated by BR at 3 h, but was also responsive to ACC, GA and IAA. It is interesting that ABA and JA had a negative effect on *BRRLK* at 3 h.

Preliminary insight into abiotic stress responses of the select genes was obtained using AtGenExpress data (Figure 4.14). All 4 genes showed transcriptional response to two or more dehydration-related stresses (osmotic, drought or cold) and UV-B. These results support our hypothesis of possible stress-related functions of these genes.

#### *4.3.9c RT-PCR analysis of candidate genes*

Since BR effects on gene expression are relatively slow and can take as long as 18-48 hrs even in short-term treatments (Zurek et al., 1994; Dhaubhadel and Krishna, 2008), we tested the expression of candidate genes in leaf tissues by RT-PCR in response to 12 h treatment with EBR to study the short-term effects of BR treatment. As expected the transcript levels of *WRKY17* were suppressed in EBR-treated seedlings, whereas *WRKY33, ACP5* and *BRRLK* showed enhanced transcript levels in response to EBR (Figure 4.15).

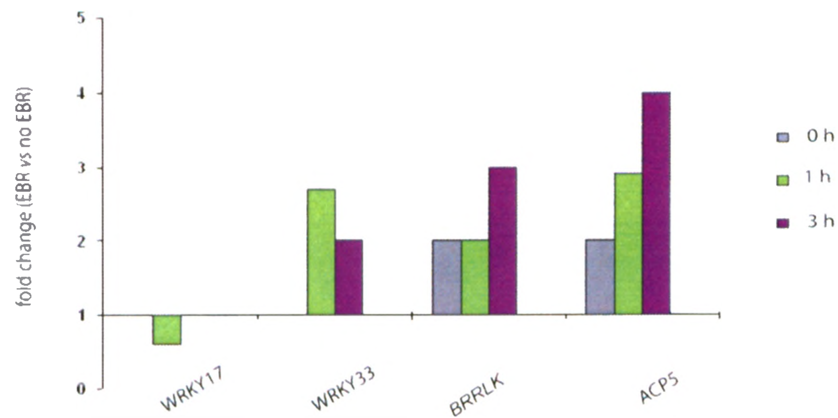
#### *4.3.9d Insertional mutant analysis*

**Characterization of T-DNA knockout lines**

A huge repository of Arabidopsis T-DNA insertional mutant lines generated by various laboratories is maintained at ABRC. The usefulness of T-DNA mutants for

**Figure 4.13** Hormone response profiles of candidate genes. A) The fold change in expression of *WRKY17, WRKY33, BRRLK* and *ACP5* in response to EBR at 0 h (no-stress), 1 h and 3 h (HS) time points. *WRKY17* was down-regulated at 1 h, *WRKY33* was up-regulated at 1 h and 3 h, *ACP5* and *BRRLK* were up-regulated at 0 h, 1 h and 3 h time points. B) The mean-normalized expression values of *WRKY17, WRKY33, BRRLK* and *ACP5* in response to 0.5 h, 1 h and 3 h treatment with different hormones generated by AVT.

A

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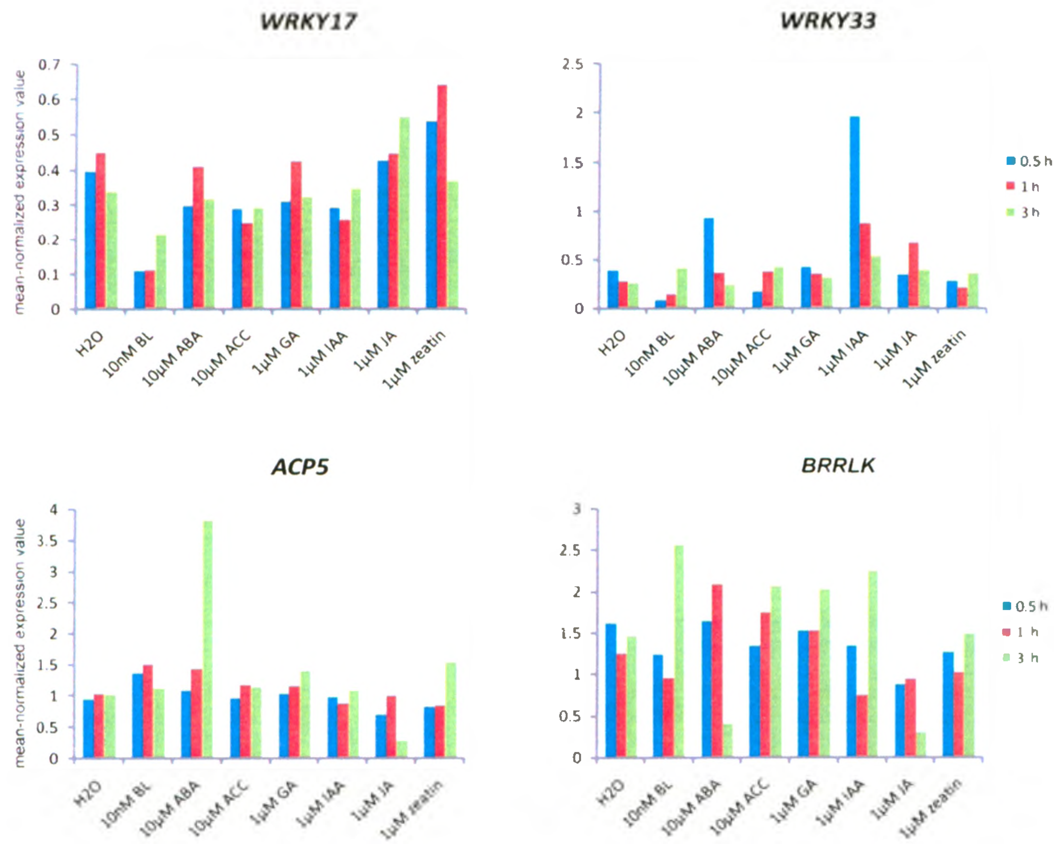

**Figure 4.14** Abiotic stress response profiles of candidate genes. The mean-normalized expression values of *WRKY17, WRKY33, BRRLK* and *ACP5* in response to time course exposure to different abiotic stresses generated by AVT.

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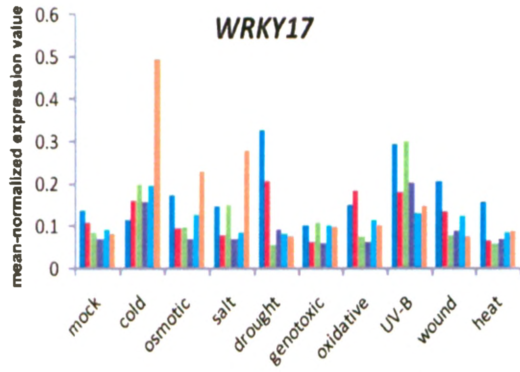
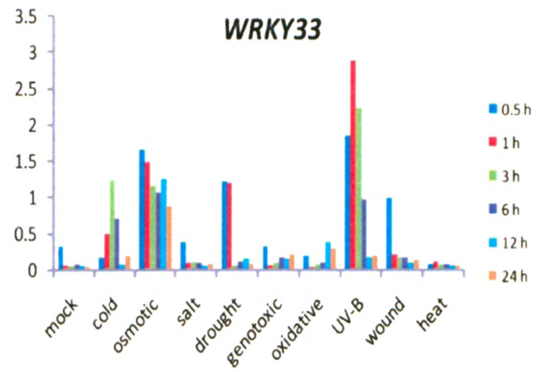
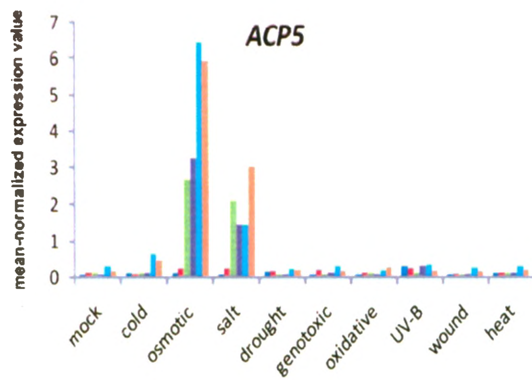

WRKY17
mean-normalized expression value
0.6
0.5
0.4
0.3
0.2
0.1
0
mock
cold
osmotic
salt
drought
genotoxic
oxidative
UV-B
wound
heat


WRKY33
3.5
3
2.5
2
1.5
1
0.5
0
mock
cold
osmotic
salt
drought
genotoxic
oxidative
UV-B
wound
heat
0.5 h
1 h
3 h
6 h
12 h
24 h


ACP5
mean-normalized expression value
7
6
5
4
3
2
1
0
mock
cold
osmotic
salt
drought
genotoxic
oxidative
UV-B
wound
heat

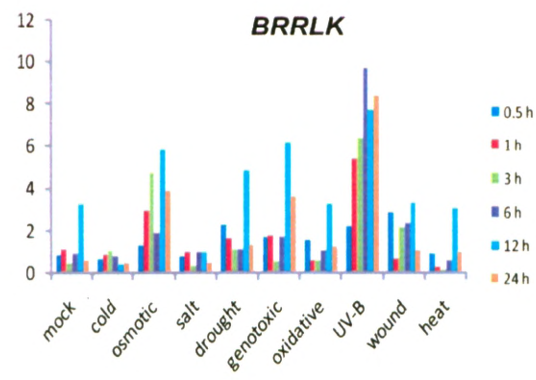

BRRLK
12
10
8
6
4
2
0
mock
cold
osmotic
salt
drought
genotoxic
oxidative
UV-B
wound
heat
0.5 h
1 h
3 h
6 h
12 h
24 h

**Figure 4.15** Effect of short-term EBR treatment on the expression of candidate genes. Wild-type (WT) seedlings grown for 21 days in the absence of EBR were treated with 1 μM EBR (E) or 0.01% ethanol (C) for 12 h. Transcript levels *WRKY17*, *WRKY33*, *BRRLK* and *ACP5* were analyzed by RT-PCR. Expression of *ACTIN* was used as loading control.

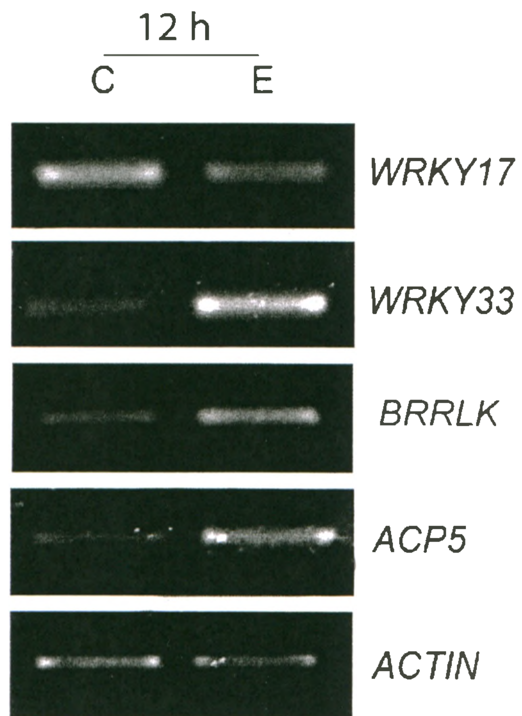

12 h
C
E
WRKY17
WRKY33
BRRLK
ACP5
ACTIN

**Figure 4.16** Characterization of T-DNA mutant lines of the candidate genes. **A)** Verification of *wrky17.1, wrky33, brrlk* and *acp5* homozygous lines for the presence of WT and mutant alleles in the genomic DNA using T-DNA specific and corresponding gene specific primers. The presence of T-DNA specific band in all the mutants (M) is shown in the left panel (INSERT), the corresponding band was absent in respective WT controls. The gene specific band was absent in all the mutants (M) and present in respective WT controls as shown in the right panel (GENE). **B)** RT-PCR confirmation of the homozygous T-DNA lines. Transcripts of *WRKYY17, WRKY33, BRRLK* and *ACP5* were not detected in the corresponding mutants (M), but were present in respective WT controls. Expression of *ACTIN* was used as control.

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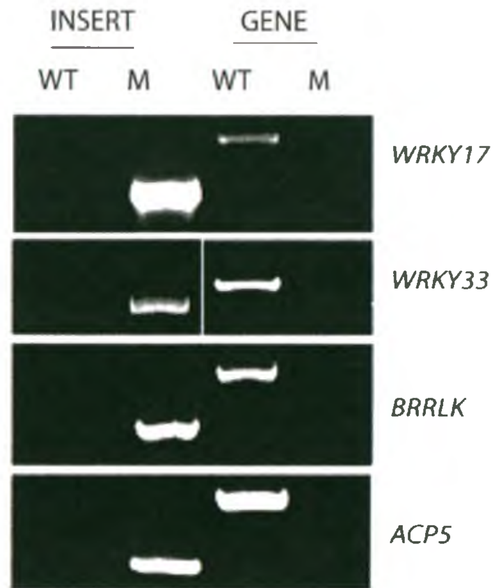
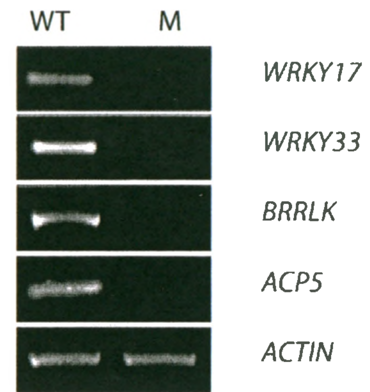
A




B



functional analysis of corresponding genes is evidenced abundantly in published literature (Krysan et al., 1999). T-DNA lines for the candidate genes were obtained from ABRC. The *WRKY17* T-DNA line (*wrky17.1*) was obtained from Dr. Thomas Kroj (Laboratory of Plant-Microorganism Interactions, France). Homozygous lines were derived by selfing and were confirmed by PCR-based genotyping. Genomic PCR using a T-DNA specific primer (LBa1 or LBb1) and gene specific primers (LP and RP) confirmed the presence of the insert and absence of gene-specific bands in all mutant lines (Figure 4.16A), indicating homozygous insertion. RT-PCR analysis confirmed that transcript expression from these genes was abolished in homozygous lines (Figure 4.16B). These homozygous lines were used in stress treatments.

**Heat stress phenotypes of insertional mutants**

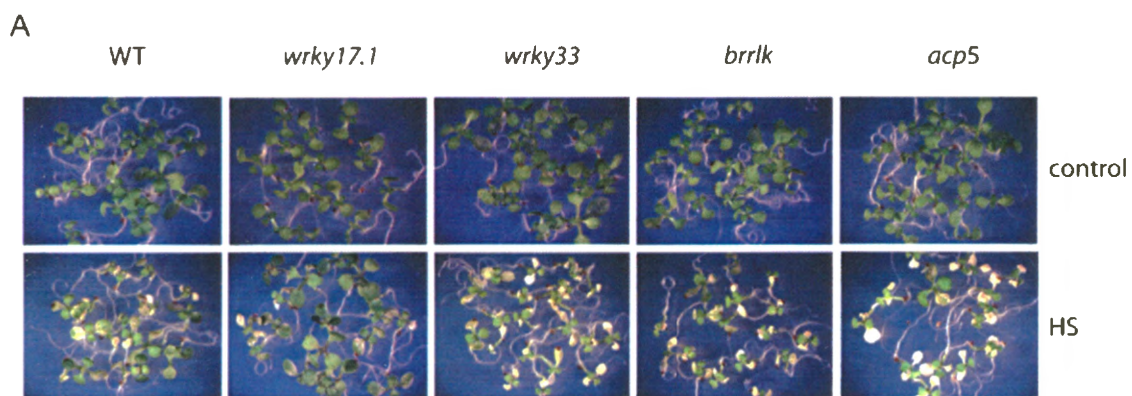
The basal thermotolerance of mutant and WT seedlings was studied by exposing 5-day-old seedlings to $44^0$C for 90 min and allowing them to recover at $22^0$C for 7 days (Liu et al., 2007). A higher percentage of WT seedlings (62%) survived the heat treatment as compared to 32% of *wrky33*, 22% of *brrlk* and 25% of *acp5* seedlings (Figure 4.17). *wrky17.1* performed slightly better (69%) than WT, but the difference in between WT and *wrky17.1* was not significant in this assay (Figure 4.17B). Since *WRKY17* is down-regulated by EBR (Figure 4.13) and is a known negative regulator of defense responses in Arabidopsis (Journot-Catalino et al., 2006), further studies are required to determine whether it performs a significant function during heat stress.

**Salt stress phenotypes of insertional mutants**

Salt conditions inhibit seed germination. T-DNA mutant and WT seeds were grown in the presence of 150 mM NaCl. Interestingly, while all genotypes germinated equally, only 30%, 12%, and 40% of the germinated seedlings of *wrky33*, *brrlk* and *acp5*, respectively, survived at 15 days after sowing seeds on the medium, as compared to the 55% survival of WT seedlings (Figure 4.18). Similar to its relatively higher thermotolerance, *wrky17.1* also showed higher percent survival (60%) on salt (Figure 4.18B). These data indicate that WRKY33, BRRLK and ACP5 do have a role in

**Figure 4.17** Heat stress phenotypes of T-DNA mutant lines of candidate genes. **A)** WT, *wrky17.1, wrky33, brrlk* and *acp5* seedlings grown for 5 days were subjected to HS at 44$^0$C for 90 min and returned to growth chamber for recovery. Plant pictures were taken 3 days after recovery. **B)** Percent survival was calculated by scoring the number of seedlings survived after 7 days of recovery. Seedlings that showed bleaching were considered dead and those with green leaves were considered surviving. All experiments were performed in triplicates with $n > 30$. Error bars represent standard error (SE) of mean for three replicates. The threshold of significance as analyzed by student's t-test is indicated above the histograms ($*p < 0.02$).

A

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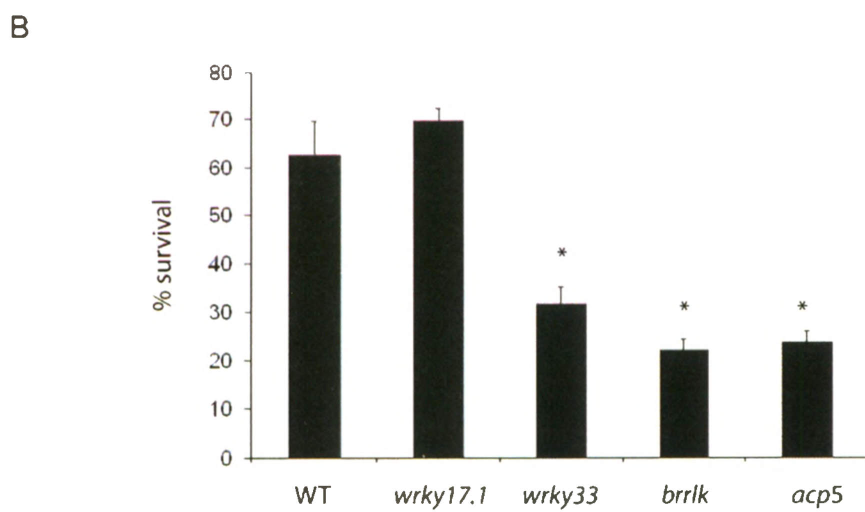
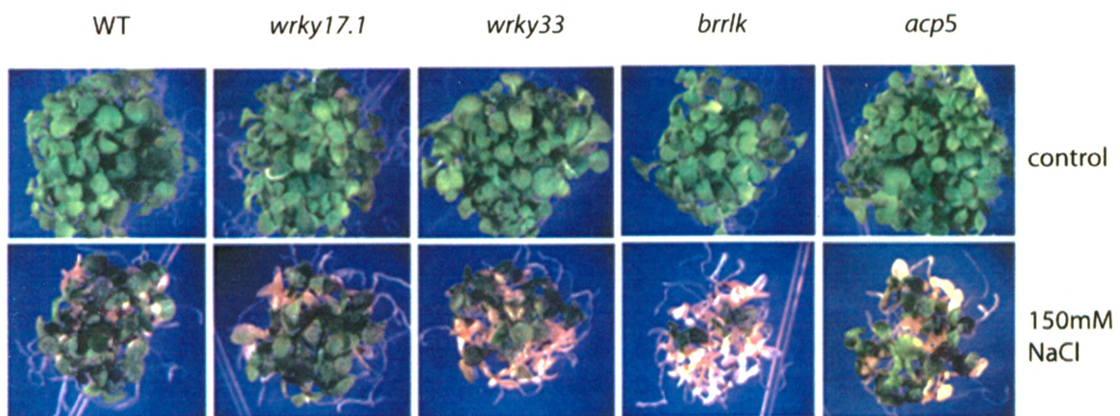
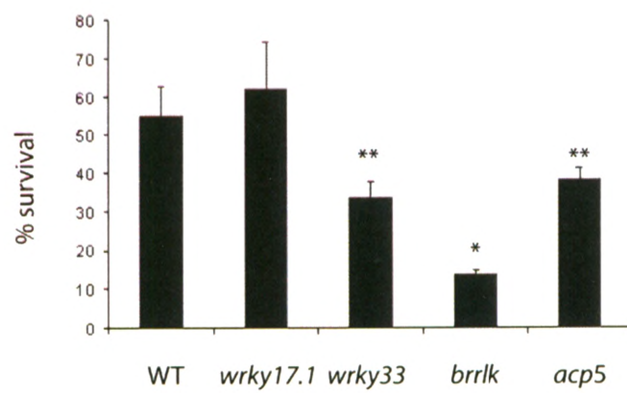


**Figure 4.18** Salt stress phenotypes of T-DNA mutant lines of candidate genes. **A)** WT, *wrky17.1, wrky33, brrlk* and *acp5* were allowed to grow on medium in the presence or absence of 150 mM NaCl. **B)** Percentage of seedlings surviving on 150 mM NaCl was calculated by counting the number of seedlings that showed true leaves and green colour at 15 days after imbibition. All experiments were performed in triplicates with $n > 30$. Error bars represent SE of mean for three replicates. The threshold of significance as analyzed by student's t-test is indicated above the histograms (*$p$ =0.01, **$p$ =0.09).

A




B



combating salt stress in germinating seedlings, the role of WRKY17 in this is less clear. While our work was in progress, a report demonstrating involvement of WRKY33 in salt stress responses appeared in literature (Jiang and Deyholos, 2009).

The results of stress treatments together indicate that three of the four genes selected for functional analysis are involved in heat and salt stress responses of Arabidopsis. This study is therefore considered successful in identifying new 'BR response genes' that have roles in stress tolerance.

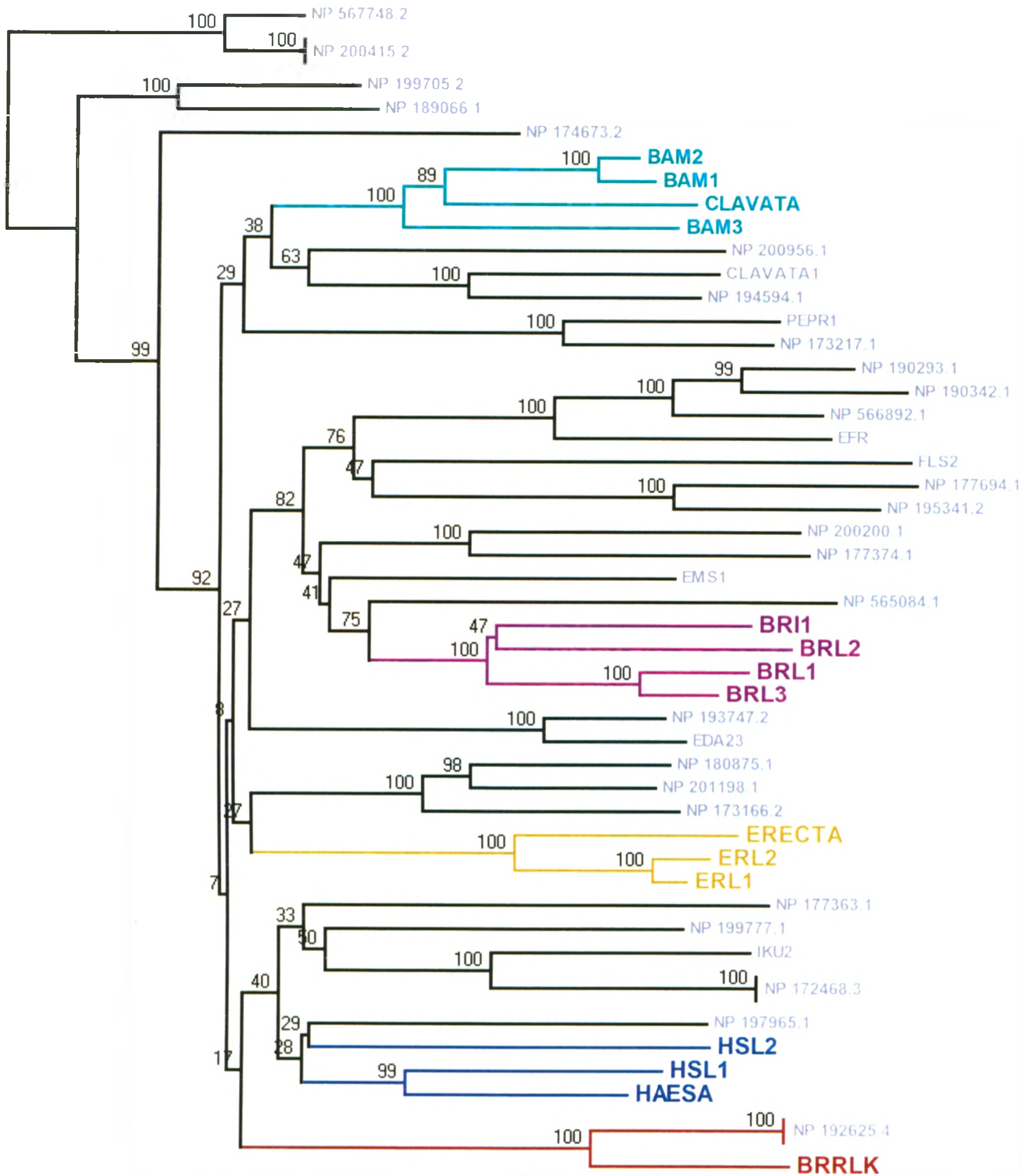
### *4.3.10 Phylogenetic analysis of BRRLK, a novel BR response gene*

Since *BRRLK* was identified as a novel BR response and stress-related gene in this study, we focused on learning more about this gene through *in silico* analyses. A PSI-BLAST search with the BRRLK protein sequence retrieved > 50 RLKs. Phylogenetic analysis using the first 50 BLAST matches resulted in the identification of a single most closely related protein (NP_192625.4) with 59% amino acid identity to BRRLK (Figure 4.19), which is known to change phosphorylation states in response to a pathogen elicitor (Benschop et al., 2007). The closest clade to BRRLK and NP_192625.4 consists of HAE (HAESA), HSL1 (HAESA-LIKE1) and HSL2 (HAESA-LIKE2) proteins, which are known to be involved in abscission of floral organs (Stenvik et al., 2008). The previously characterized and functionally related RLKs (colored branches in Figure 4.19) were grouped into distinct clusters indicating their relatedness. Known RLKs involved in BR signaling, such as BRI1 and its homologues BRL (BRI1 LIKE) 1, 2 and 3, were grouped together in a distant clade indicating that BRRLK is not closely related to this gene family. BAK1, a BR co-receptor, was not picked among the first 50 homologues. Thus, *BRRLK* is a novel 'BR response gene' with putative functions in pathogen defense and cell-death.

## 4.4 DISCUSSION

Genes responsive to BR within short periods of treatment have been identified in genome-wide microarray studies (Goda et al., 2002, 2004; Müssig et al., 2002; Yin et al., 2002; Nemhauser et al., 2004), and although there were similarities in the functional

**Figure 4.19** Phylogenetic analysis of BR RESPONSIVE RECEPTOR LIKE KINASE (BRRLK) and related proteins in Arabidopsis. BRRLK related proteins were identified by PSI-BLAST search. The first 50 sequences retrieved were aligned by the multiple sequence alignment program CLUSTALW 2.0. Phylogenetic tree was generated by the neighbour-joining method using MEGA4 phylogenetic software package. Bootstrap values (shown at the corresponding nodes) were obtained from 1000 replicates and are reported as percentages. Colored clades indicate the grouping of functionally related proteins.


NP 567748.2
NP 200415.2
NP 199705.2
NP 189066.1
NP 174673.2
BAM2
BAM1
CLAVATA
BAM3
NP 200956.1
CLAVATA1
NP 194594.1
PEPR1
NP 173217.1
NP 190293.1
NP 190342.1
NP 566892.1
EFR
FLS2
NP 177694.1
NP 195341.2
NP 200200.1
NP 177374.1
EMS1
NP 565084.1
BRI1
BRL2
BRL1
BRL3
NP 193747.2
EDA23
NP 180875.1
NP 201198.1
NP 173166.2
ERECTA
ERL2
ERL1
NP 177363.1
NP 199777.1
IKU2
NP 172468.3
NP 197965.1
HSL2
HSL1
HAESA
NP 192625.4
BRRLK
0.1

categories of the identified genes, there was little overlap in genes identified in different studies. The differences were attributed to the different genotypes (Müssig and Altmann, 2003), experimental conditions and analytical methods used in these studies (Vert et al., 2005). Furthermore, the earlier Affymetrix arrays represented only about one third of the Arabidopsis genome. Realizing the limitations of the earlier studies, Vert et al. (2005) performed a combined analysis of the data from these studies together with the data generated using the Affymetrix ATH1 whole-genome array (approximately 22,000 genes) to generate a high confidence list of BR-regulated genes. The predominant categories in this list are genes involved in the biogenesis and modification of cell wall, production and secretion of long chain fatty acids, biosynthesis and/or signaling of phytohormones, cytoskeleton genes, and transcription factors (Vert et al., 2005).

In recent years, the role of BR in plant stress responses has been established at the molecular level (Divi and Krishna, 2009; Dhaubhadel et al., 1999; 2002; Kagale et al., 2007; Szekeres, 2003). Targeted and non-targeted investigations of the molecular changes associated with BR-mediated increase in abiotic stress tolerance have indicated that BR regulates gene expression at both transcriptional and post-transcriptional levels (Dhaubhadel et al., 1999; 2002; Kagale et al., 2007; Dhaubhadel and Krishna, 2008). Since it would be useful to separate the changes impacted by BR alone from those brought about by stress alone or by stress and BR together, we analysed transcriptional changes using Affymetrix ATH1 arrays in response to EBR under no-stress (0 h), and HS (1 h and 3 h at $43^0$C), as well as in response to HS alone (1 h and 3 h at $43^0$C). Few assumptions were made prior to the analysis of the data. Since seedlings used in the present study were grown for 21 days in the presence of EBR, it was assumed that both primary and secondary BR response genes would be detected in the study. Based on our experimental design, it was expected that the differentially-regulated genes would fall in the following categories: 1) HS-responsive, 2) BR+HS-responsive and 3) BR-responsive (under no-stress and/or HS conditions). A two-way approach was used to identify differentially-regulated genes. The first two category genes were identified by analyzing gene expression changes in response to EBR alone (0E), HS alone (1C and 3C) and EBR+HS (1E and 3E) treatments as compared to no-EBR and no-stress (0C) control. The

third category genes were identified by comparing gene expression of EBR-treated (E) with untreated (C) control at different HS time points (0 h, 1 h and 3 h).

#### *4.4.1 BR and HS: Relating expression patterns with biological functions*

To understand the effects of EBR, HS and EBR+HS treatments, hierarchical clustering was performed on genes responding to all these treatments in comparison with no-EBR and no-stress (0C) controls. Genes from each cluster were analyzed for enriched GO terms and promoter motifs. In a reverse approach, genes were grouped according to their functions by MapMan analysis and then related to the clusters. Clusters 2 and 5 were regulated similarly by HS and EBR, and the combination of EBR+HS had an additive effect (Figure 4.3). While cluster 2 genes were down-regulated, cluster 5 genes were up-regulated by all the treatments. GO analysis showed that cluster 5 genes are predominantly localized to the cytoplasm and related to stress tolerance, regulation of metabolism and signaling, while cluster 2 genes belong to chloroplast and were related to electron transport. Similar patterns were observed by MapMan analysis (Figure 4.4). The stress and signaling bins were enriched with cluster 5 genes, while the RNA bin was enriched with cluster 2 genes. Three genes with demonstrated stress-related functions in cluster 5 include *ATHSFA4A*, *ERD14* and *RPS2*. *AtHSFA4A* has been linked with early stages of oxidative stress acclimation (Daveltova et al., 2005). ERD14 is a member of the dehydrin family with chaperone activity. ERD14 and its family member ERD10 contribute to preventing heat-induced aggregation of proteins (Kovacs et al., 2008). We have previously shown that *ERD10* expression is increased by BR treatment under dehydration conditions (Kagale et al., 2007). In the current study ERD10 was placed in cluster 9. The genes in cluster 9 were highly up-regulated by HS and EBR+HS, but only slightly up-regulated by EBR alone.

Another important functional group of cluster 5 genes is hormone metabolism. Majority of these genes are related to JA and ET, including *CEJ1 (COOPERATIVELY REGULATED BY ETHYLENE AND JASMONATE 1)*, *AOC4 (ALLENE OXIDE CYCLASE 4)*, *JR1 (JASMONATE RESPONSIVE 1)* and *ATERF15 (ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 15)*. The roles of these hormones in stress responses are sufficiently documented in literature (Clarke et al., 2009; Alonso et al.,

1999; Balbi and Devoto, 2008). These results emphasize the roles of ET and JA pathways in BR-mediated increase in thermotolerance and warrant investigation of the levels of these hormones following BR treatment.

The RNA bin genes from cluster 2 mostly included different types of transcription factors, including ARFs and ARRs. Genes belonging to the *ARF* and *ARR* families such as *ARF6, ARF19, ARR4, ARR7, ARR15* and *ARR16* were all down-regulated by the different treatments. *ARF19* and *ARR15* were not included in the 'BR response genes' category as their expression changes > 2-fold only in EBR+HS condition as compared to no-EBR and no-stress (0C) control. Down-regulation of *ARFs* and *ARRs* by BR was a common outcome of all previous BR response studies (Vert et al., 2005). Previously, *ARF19*, *ARF6, ARR3, ARR5* and *ARR6* were detected in this context. Thus, *ARF19* and *ARF6* were down-regulated by BR in previous as well as our study. It is interesting to note that these genes were also down-regulated by HS and EBR+HS. *ARRs* encode transcriptional repressors that are induced by cytokinin treatment and are involved in negative feed-back regulation of the cytokinin pathway (Suzuki et al., 2004). Since different gene members of the *ARR* gene family were identified in previous and current studies, we concur with the conclusion of Vert et al. (2005) that while BR responses point to the same functional category of genes, some differences are observed due to variations in experimental conditions.

Gene clusters 1 and 7 are interesting in that both were highly up-regulated by EBR alone and down-regulated by HS alone and by EBR+HS. Functional terms over-represented in these clusters relate to endomembrane system, cell wall, phytohormone JA, and lipid transport and binding. These are some of the known BR responses (Müssig and Altmann, 2003; Vert et al., 2005) related to growth and development, which in general are down-regulated by HS (Larkindale and Vierling, 2008). While BR treatment maintains this trend, down-regulation of cluster 7 genes was less pronounced under the EBR+HS combination, presumably due to a lower impact of stress on EBR-treated seedlings. Within this set, JA biosynthesis genes *LOX2* and *OPR3* were included, which have also been noted before as BR response genes (Müssig et al., 2002; 2006; Müssig and Altmann, 2003). Thus, a strong connection between BR and JA has been reinforced by our study. Taking the example of these and other such genes that have emerged as

BR-responsive in previous and current studies, we establish that both short-term and long-term treatment with BR can lead to the identification of primary response genes. It is a short-sighted view to assume that long-term treatment with BR is likely to deliver only second response genes.

Cluster 8 genes were regulated in opposite directions by EBR and HS, however, unlike clusters 1 and 7 genes, which were predominantly regulated by HS, cluster 8 genes were predominantly regulated by EBR. Enrichment of clusters 8 and 9 with ABRE-like binding site motif and functional terms related to abiotic stresses indicates that ABA and abiotic stress responses are induced by BR under both normal and HS conditions. The cluster 8 genes include ABA-responsive genes with documented roles in stress tolerance, such as *KIN1, RD29A* and *COR15A* (Kurkela and Franck, 1990; Yamaguchi-Shinozaki and Shinozaki, 1993; Baker et al., 1994; Steponkus et al., 1998), and LTPs with possible roles in cell-wall loosening Nieuwland et al. (2005), lipid transport, cutin deposition, embryogenesis, pathogen defense and adaptation to environmental conditions (Kader, 1996). The expression patterns of this set of genes point to extensive cross-talk between BR and ABA in conferring stress tolerance. An important point of consideration is that several stress-responsive genes up-regulated by BR in the present study are either not affected by HS or down-regulated by HS. It is tempting to speculate that the induction of these genes by BR under no-stress and HS may contribute to enhanced thermotolerance in treated seedlings.

Cluster 9 genes were the second major category in the MapMan stress bin and include stress-responsive *HSF4, HSP70-2*, a DNAJ family member, *ERD10, COR47, LTPs* and the universal stress protein (USP) family genes. These genes were highly up-regulated by HS, while EBR alone had little to no effect on these genes. However, EBR greatly augmented their stress-responsive expression (EBR+HS). Thus, EBR contributes to stress tolerance in plants in more than one way.

A majority of the genes responding to EBR, HS and EBR+HS were related to protein fate and were regulated similarly by HS and EBR. It has been shown that both protein synthesis and degradation are important in producing stress tolerance (Huang and Xu, 2008; Huffaker, 1990). Several genes involved in protein synthesis and degradation were repressed (group E; Figure 4.5), while a majority of genes involved in post-

translation modification and protein degradation were induced (group E and D; Figure 4.5). In both groups of genes the effect of EBR+HS was more pronounced as compared to HS or EBR alone (Figure 4.5). A role for BR in protein post-translational modification has emerged from this study and warrants further investigation. It is possible that BR's control of protein fate and activity through post-translational modifications overrides its modest transcriptional changes in controlling myriads of cellular processes.

A majority of group A (induced by EBR alone; repressed by HS and EBR+HS) and B (induced by EBR alone and EBR+HS; repressed by HS alone) genes were involved in protein degradation. Enhanced expression of proteases, mainly cysteine proteases, which were also evident in the present study, is linked with exposure to high temperature, drought and salinity (Callis, 1995). A role for cysteine proteases in thermotolerance and regulation of cell-death during senescence has been suggested (Huang and Xu, 2008; Chen et al., 2002). Enhanced expression of these genes by BR would likely add to this role, which in turn may impact superior thermotolerance or better performance of treated seedlings.

### *4.4.2 BR response genes*

In order to identify BR response genes involved in stress tolerance, genes responding to EBR treatment under no-stress (0E *vs.* 0C) and HS conditions (1E *vs.* 1C and 3E *vs.* 3C) were analyzed. Venn diagram analysis of BR response genes revealed that while a majority of genes are responsive to BR under all conditions, certain genes were unique to either no-stress or 1 h of HS or 3 h of HS and some genes were common to any two time points (Figure 4.6A and B). GO analysis revealed that BR has a major induction effect on processes related to stress and transport; more genes were induced by BR than repressed in these categories. Genes involved in processes like cell organization/biogenesis, development, transcription, protein metabolism and signal transduction were either induced or repressed by BR. The electron transport/energy and DNA or RNA metabolism pathways were only repressed. The latter would likely be beneficial under HS conditions. Taken together, this analysis revealed major induction of stress-related responses by BR, while the regulatory processes were either induced or repressed to suit the existing conditions.

Abiotic stress signaling in plants involves receptor-coupled phosphorelay, phosphoinositol-induced calcium ($Ca^{2+}$) changes, mitogen-activated protein kinase cascades and transcriptional activation of stress-responsive genes (Xiong and Zhu, 2001). In addition, regulation of oxidative states, protein post-translational modifications, hormone biosynthesis and signaling are also important. Significant changes in response to BR were observed in transcripts related to all these processes (Figure 4.7).

In plants, $Ca^{2+}$ acts as a second messenger during abiotic stress signaling (Knight, 2000). Transient increase in cytosolic $Ca^{2+}$ by HS was observed in Arabidopsis cell cultures and a role for $Ca^{2+}$ and calmodulin (CaM) in the expression of hsps has been proposed (Liu et al., 2007). A total of five $Ca^{2+}$ signaling genes were identified in this study as BR-responsive, including the stress-responsive *RD20.* RD20 is a $Ca^{2+}$-binding protein induced by drought, ABA and high salinity (Takahashi et al., 2000). It should be noted that the BR biosynthesis enzymes DWF1, DWF4 and CPD contain $Ca^{2+}$/CaM-binding domains, and $Ca^{2+}$/CaM signaling was found to be critical for the function of DWF1 (Du and Poovaiah, 2005). Hence, it is plausible that endogenous BR levels are modulated by stress or by exogenous BR through changes in cellular $Ca^{2+}$. Whether or not the BR-responsive $Ca^{2+}$ signaling genes are involved in moduating BR levels or stress responses remains to be seen.

The role of MAP kinases in hormone and stress signaling pathways is well established (Nakagami et al., 2005). MAP kinases like *MPK3* and *MEK1* with known stress-related roles (Droillard et al., 2002; Rentel et al., 2004; Xing et al., 2007) were identified as BR-responsive in this study. Transcription factors are a major group within BR response signaling genes. Vert et al. (2005) reported transcription factors to constitute > 10% of the BR down-regulated genes. In the current study 50% of BR-responsive transcription factors were down-regulated. Our finding that several WRKY transcription factors are BR-responsive is notable. WRKY transcription factors are known key regulators of plant defense responses, however their roles in abiotic stress responses are not well described (Eulgem et al., 2000). Recent studies have established WRKY70 as an important component of plant senescence, disease resistance and SA signaling (Ulker et al., 2007; Li et al., 2004; 2006; Ren et al., 2008). In Chapter 3 we have shown that BR

treatment enhances *WRKY70* transcript levels even in the biosynthesis and signaling mutants of SA, indicating possible direct regulation by BR of either *WRKY70* itself or an upstream component of SA (Chapter 3). WRKY33 has been associated with various pathogen defense responses (Lippok et al., 2007; Zheng et al., 2006), and more recently, with salt stress (Jiang and Deyholos, 2009). Both *WRKY70* and *WRKY33* were up-regulated by BR, while *WRKY17*, a negative regulator of defense responses (Journot-Catalino et al., 2006) was down-regulated in our microarray study. Since WRKY factors are transcriptionally activated via MAP kinase signaling cascades (Kim and Zhang, 2004), it is tempting to speculate that BR regulation of MAP kinases may impact expression changes of WRKY and other transcription factors, culminating in the expression of stress- and hormone-responsive genes.

Other BR response genes consisted of those involved in oxidative stress, protein and hormone metabolism. Genes involved in cell detoxification, such as *glutathione S-transferases* (GSTs) and *glutaredoxins* (GRXs) (Edwards et al., 2000; Rouhier et al., 2008) are down-regulated during the acquisition of thermotolerance (Larkinadle and Vierling, 2008). In line with this, six glutaredoxin (GRX) genes were down-regulated by BR. *CAT3* belonging to the protein functional category of catalases that are involved in $H_2O_2$ scavenging was up-regulated by BR. *CAT3* expression can be induced by drought, salt and ABA, and its induction by ABA is dependent on MEK1 (Xing et al., 2007), a MAP kinase found also to be induced by BR in our study. Though far-fetched at this point in time, BR-induced ABA levels may drive the expression expression of *MEK1* and *CAT3,* which may ultimately contribute to protection against oxidative damage, as evidenced in Chapter 3.

Genes involved in post-translational protein modification include several protein kinase family genes, two protein phosphatase 2C (PP2C) family genes and a *MAPKKK10*. The PP2C gene family is one of the largest families identified in plants and induced expression of several of the family members by diverse stimuli indicates their primary role in stress tolerance, especially ABA response (Xue et al., 2008). Numerous connections of BR with ABA-mediated stress tolerance mechanisms have emerged in this

study. The challenge for the future is to determine whether BR works via modulation of ABA levels or shares overlapping targets with ABA.

In addition to ABA marker genes, BR also induces the expression of SA, JA and ET marker genes, even in the corresponding hormone mutants (Chapter 3). Reminiscent of these are the SA-responsive genes *PR-1 (PATHOGENESIS-RELATED GENE 1)* and *WRKY70*, JA marker gene *LOX2*, and ABA marker gene *RD22*. The present study found *WRKY70*, *LOX2* and other ABA-responsive genes *RD20* and *KIN1* to be induced by BR under both no-stress and HS conditions (Figure 4.7 and 9). The up-regulation of several stress and disease-related genes such as *HSF4, ATHSFA4A, ERD10, ATOSM34, COR15A, ATGSTF6/GST1, PR4, RPS2, AtCHIB, TIR-NBS-LRR*s by BR even under no-stress conditions likely makes the plant more prepared to combat stress, but what may be the downside of expression of these genes under no stress conditions is currently not known.

To gain insight into possible stress-related roles of BR response genes, their stress and hormone response profiles were analyzed by AVT. For stress response profiles, the complete BR response gene list (0 h, 1 h and 3 h) was analyzed; whereas for hormone response profiles BR *only* genes (responsive to BR under no-stress [0 h] condition) were analyzed in order to remove the effect of HS. BR response genes were found to be regulated by several abiotic stresses like drought, salt and osmotic stress, while HS did not seem to have much effect (Figure 4.10A). One possible explanation could be the difference in the HS treatments; exposure to $38^0$C for 3 h (Kilian et al., 2007) is milder as compared to $43^0$C for 3 h (our study). Another possible explanation could be that exogenous BR evokes a general stress response in plants. Indeed, genes responsive to cold, salt and osmotic stresses share core genomic response (Kilian et al., 2007). Another important finding in this part of the analysis was that similar to the findings of GO and promoter motif analyses, a majority of BR response genes were highly responsive to ABA and JA. Thus, a collaborative relationship between BR and ABA in modulating stress responses has clearly emerged from this study. At the same time, antagonistic relations between BR and ABA are also clear; 7 out of 8 genes maximally up-regulated

by BR as compared to other plant hormones (Figure 4.11A), were down-regulated by ABA.

The positive impact of BR on sucrose-starch and secondary metabolism under no-stress and HS conditions (Fig 8A and B) is also worthy of comment. It has been suggested that simple sugars are better osmoprotectants, preventing cellular membranes from damage caused by dehydration and freezing (Ma et al., 2009; Shao et al., 2006), and contributing to the protection of photosynthetic electron transport chain, stromal proteins, and chloroplast membranes during freezing stress (Kaplan and Guy, 2005). Phenylpropanoid compounds like flavonoids, lignins and anthocyanins function in plant defense against biotic and abiotic stresses. Flavonoids act as antioxidants and plant protectants against pathogens and abiotic stresses, including HS and drought (Pourcel et al., 2007; Coberly and Rausher, 2003; Warren and Mackenzie, 2001). Lignin accumulation is an important component of the cell wall thickening process (Shirley, 1996). Anthocyanin accumulation protects dehydrated plants from photoinhibition (Balakumar et al., 1993) and freezing stress (McKown et al., 1996). These examples make a case for BR contributing to stress tolerance via these compounds and their associated mechanisms.

### *4.4.3 Stress–related functions of new BR response genes*

A subset of BR response genes (*WRKY33, WRKY17, ACP5* and *BRRLK*) were characterized for abiotic stress-related roles. WRKY33 and WRKY17 have been implicated in pathogen defense (Zheng et al., 2006; Journot-Catalino et al., 2006) and ACP5 in phosphate mobilization and metabolism of reactive oxygen species (del Pozo et al., 1999). *BRRLK* is a previously uncharacterized *LRR-RLK* gene that is maximally up-regulated by BR (Figure 4.11A). Analysis of T-DNA insertion mutants *wrky33, acp5* and *brrlk* revealed lower survival rates for these mutants under heat and salt stress conditions, as compared to WT and *wrky17.1*. During the course of our study, a report describing a possible role for WRKY33 in salt stress appeared in literature (Jiang and Deyholos, 2009). While *wrky33* plants were moderately sensitive to salt as compared to WT, overexpression of *WRKY33* was sufficient to increase salt tolerance in Arabidopsis (Jiang and Deyholos, 2009). These results validate our findings for WRKY33.

*WRKY17* was chosen for preliminary functional analysis since it was down-regulated by BR in our study and it is a known negative regulator for defense responses (Journot-Catalino et al., 2006). The lack of any significant phenotypes under our experimental conditions suggests that either *WRKY17* has little connection with salt and heat tolerance or that alterations in experimental conditions are required to produce a detectable phenotype.

The most significant finding under this analysis was *BRRLK*. Mutant analysis clearly indicated a function for *BRRLK* in heat and salt stress tolerance. Based on the close association of BRRLK and its closest homologue NP_19625.4 (At4g08850) with HEASA, HSL1 and HSL2, which are involved in floral abscission (Figure 4.19; Stevik et al., 2008), and the finding that NP_19625.4 phosphorylation states change upon treatment with pathogen elicitor (Benschop et al., 2007), we hypothesize a role for BRRLK and NP_19625.4 in cell-death and defense responses. The BR co-receptor, BAK1, participates in cell-death and defense responses by interacting with other LRRs (Chinchilla et al., 2007; He et al., 2007; Kemmerling and Nürnberger, 2008). Future studies involving overexpression in transgenic plants, pathogen and elicitor responses, and protein-protein interactions may reveal functions of this novel BR response gene, if any, in defense and cell-death responses.

In summary, our genome-wide expression analysis has unveiled the many mechanisms probably operated by BRs in modulating plant stress responses; these include cross-talk with ABA and JA, calcium signaling, post-translational modifications of proteins, cellular redox, and cellular metabolism involving carbohydrate metabolism and accumulation of phenylpropanoid compounds. These mechanisms would enable structural and physiological adjustments required for plant survival under adverse conditions. The next challenge is to find how BR controls these mechanisms.

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# CHAPTER 5

## Overexpression of *AtDWARF4* in Arabidopsis seeds increases cold tolerance in seedlings and overcomes ABA-induced inhibition of germination

# CHAPTER 5

## 5.1 INTRODUCTION

Brassinosteroids (BRs) are a group of naturally occurring plant steroidal compounds with wide ranging biological activity. Due to the substantial effects of BRs on plant growth and development, the economic potential of BRs in agriculture was recognized as early as the 1980s. Extensive testing of a synthetic BR, 24-epibrassinolide (EBR), in China, Japan and Russia showed that while exogenous BR has the ability to substantially increase yield in a variety of plant species, the results can be variable depending on the mode of application, growth stage at time of application, and the environmental conditions (Khripach et al., 2000; Ikekawa and Zhao, 1991). The high cost of synthetic BRs together with variability in results has discouraged their use in agriculture. Since BRs control several important agronomic traits such as flowering time, plant architecture, seed yield and stress tolerance, genetic manipulation of BR biosynthesis, conversion or perception offers a unique possibility of significantly increasing crop yields through both changing plant metabolism and protecting plants from environmental stresses.

Several BR biosynthetic and signaling genes have been characterized over the last two decades (Müssig, 2005). This knowledge has been utilized in altering BR levels or perception for engineering agronomic traits in different plant species (Table1; Divi and Krishna, 2009a). The impact of manipulating the expression of a BR biosynthetic gene, *DWF4 (DWARF4),* in transgenic plants was first reported by Choe et al. (2001). The *DWF4* gene encodes a cytochrome P450 monooxygenase (CYP90B1), which mediates a rate-limiting step in BR biosynthesis (Choe et al., 1998), and is under tight transcriptional/post-transcriptional regulation that is important in maintaining BR homeostasis (Kim et al., 2006). The Arabidopsis *dwf4* (*dwarf4*) mutants are severely dwarf and display typical BR-deficient phenotypes that can be rescued by exogenous BR application (Choe et al., 1998). Ectopic overexpression of *DWF4* in transgenic Arabidopsis resulted in > 2-fold increase in the total number of branches and siliques, and 59% increase in seed yield, as compared to wild-type (WT) (Choe et al., 2001). The increase in seed production was due to greater number of seeds per plant. The transgenic

**Table 5.1** Summary of BR biosynthesis and signaling genes that were modified in expression or function in various studies.

| | Gene name | Gene description | Genetic modification | Plant species used | Observed phenotype |
|---|---|---|---|---|---|
| BR biosynthesis | *DWF4* | Arabidopsis thaliana cytochrome P450 monooxygenase | ectopic over-expression | Arabidopsis | increase in seed yield |
| | *ZmDWF4* | maize ortholog of DWF4 | ectopic over-expression | Arabidopsis | increase in seed and branch number, as well as the height of florescence stem |
| | *CYP* | C22 hydroxylase | over-expression in stem, leaves and roots | rice | increase in grain yield and photosynthetic effeciency |
| | *GhDET2* | cotton ortholog of *DET2* (steroid 5α-reductase) | seed coat-specific expression | cotton | increase in fibre yield and quality |
| | *OsDWARF4* | rice ortholog of *DWF4* | gene knock out (retro-transposon) | rice | semi dwarf; erect leaves; increase in biomass and grain yield under conditions of dense planting; small seeds |
| BR signaling | *OsBRI1* | rice ortholog of *BRI1* (BR-receptor) | co-suppression | rice | erect leaves; increase in yield potential |
| | *AtBAK1* | BR-coreceptor | ectopic overexpression | rice | semi dwarf plants |
| | *UZU (HvBRI1)* | barley ortholog of *BRI1* (BR-receptor) | single nucleotide substitution (BR-insensitive) | barley | semi dwarf; increase in yield and lodging resistance |
| | *OsGSK1* | rice ortholog of BIN2 (GSK3/ SHAGGY-like protein kinase, negative regulator of BR signalling) | gene knock out (T-DNA) | rice | increase in abiotic stress tolerance |
| Putative steroid regulation | *AtHSD1* | 11-β-hydroxysterol dehydrogenase | ectopic overexpression | Arabidopsis | increase in growth, yield, and salt tolerance; constitutive expression of BR-responsive genes |

plants also had up to 40% increase in inflorescence height at maturity as compared to WT. *DWF4* orthologs were also identified in other crops like rice and maize. Constitutive expression of *ZmDWF4* in three Arabidopsis *dwf4* mutants not only rescued the mutant phenotype to WT, but also increased the seed and branch numbers, as well as the height of florescence stem (Liu et al., 2007).

Another important step in BR biosynthesis is catalyzed by DET2 (DE-ETIOLATED 2). The *DET2* gene encodes a steroid 5α-reductase involved in BR biosynthesis (Noguchi et al., 1999). To investigate the role of BRs in cotton fiber development, the *DET2* ortholog in cotton was cloned and expressed under the control of a seed coat-specific promoter, pFBP7. Transgenic lines overexpressing *GhDET2* showed 22.6% and 10.7% increase in fiber number and fiber length, respectively, relative to control plants (Luo et al., 2007). Although this study established that BR modulation can positively affect fibre quality and yield, the fact that constitutive overexpression of *GhDET2* in transgenic cotton led to severe sterility and boll abortion suggests that altered BR levels must be optimal in order to produce a desired phenotype.

In addition to their role in plant development, BRs protect plants from a variety of environmental stresses, including high and low temperatures, drought, salinity and pathogen attack (Krishna, 2003; Divi and Krishna, 2009b). Most of the studies demonstrating anti-stress effects of BRs have employed treatment of plants with exogenous BRs. Although exogenous BR treatment is yet neither a conventional greenhouse nor field agricultural method, it is highly instrumental in the laboratory for studying BR-induced molecular changes that may contribute to increased stress tolerance in treated *vs.* untreated plants. Results of a global gene expression study of untreated and BR-treated Arabidopsis under stress and no-stress conditions suggest that BR affects expression of numerous stress-responsive genes either directly or through cross-talk with other plant hormone pathways, resulting in better performance of plants under stress conditions (Chapter 4). These results, together with the observation that T-DNA knockout mutants of *OsGSK1*, a rice orthologue of the BR negative regulator BIN2 (BRASSINOSTEROID-INSENSITIVE 2), exhibit greater tolerance to cold, heat, salt, and drought stresses as compared with non-transgenic segregants (Koh et al., 2007),

indicate that altering endogenous BR activity through transgenic technology has promise of generating crops with increased stress tolerance.

A possible role for BRs in seed development and germination has been suggested in several studies. Accordingly, rapidly growing seeds contain relatively higher levels of the biologically active BRs brassinolide and castasterone (Nomura et al., 2007). However, little is known about how endogenous BR levels correlate with seed growth, germination and stress tolerance. In oil seeds, lipids are generally stored as triacylglycerols (TAGs) in spherical compartments called oilbodies (Murphy, 1993). Olesosins are hydrophobic plant proteins that cover the surface of the oilbody and constitute about 2 – 3% of total seed protein in mature seeds (Huang, 1992). Oleosins and their transcripts accumulate in developing seeds, primarily in embryonic tissues (Huang, 1992; Plant et al., 1994). Oleosin expression is inducible by the plant hormone abscisic acid (ABA) and sorbitol (Plant et al., 1994). The Arabidopsis oleosin gene promoter is active throughout seed maturation, including the late-cotyledonary stage of embryo development (Plant et al., 1994). The seed-specific expression of oleosin, together with correlation of its accumulation with that of TAGs (Tzen et al., 1993), makes the oleosin promoter attractive for experiments involving alterations in seed lipid biosynthesis, and seed development and maturation. The oleosin promoter has also been widely used in molecular farming for transgenic expression and recovery of biologically active therapeutic proteins from seeds (Capuano et al., 2007; Nykiforuk et al., 2006).

To assess the impact of BRs on seed development, germination and stress tolerance, *AtDWF4* was overexpressed in Arabidopsis under the control of seed-specific oleosin promoter. Preliminary analysis of the resulting transgenic lines showed that germinating seedlings have increased cold tolerance and can overcome inhibition of germination by ABA. This is the first time a correlation can be made between a possible increase in endogenous BR levels and cold stress tolerance in plants. The impact of seed-specific overexpression of *AtDWF4* on embryo development and oilbody accumulation remains to be determined.

## 5.2 MATERIALS AND METHODS

### *5.2.1 Plant material and growth conditions*

Arabidopsis, ecotype Columbia, was used in this study. Seeds were surface sterilized by sequentially soaking in 75% ethanol for 1 min, rinsing twice in sterile-distilled water, soaking in 1.05% sodium hypochlorite for 20 min with stirring, and rinsing 4-5 times with sterile distilled water. The seeds were then placed in pots containing autoclaved soil and kept at $4^0$C in dark for 3 days. Later the pots were transferred to a growth chamber set at $22^0$C, 16 h day length and a light intensity of 80 μE $m^{-2}$ $s^{-1}$. When the primary inflorescences reached 3-4 cm in height they were decapitated to induce axillary bolts.

### *5.2.2 Plasmid construction and plant transformation*

The plasmid pSBS3062, containing the Arabidopsis oleosin promoter and terminator was obtained from SemBioSys Inc. (Calgary, AB). The *AtDWF4* cDNA was obtained from the Arabidopsis Biological Resource Center (clone no. U13551). The cDNA was PCR amplified using forward (*NCOI-DWF4-F:* 5' CGTTCCATGGATGTTCGAAACAGAGCATC) and reverse (*NCOI-DWF4-R:* 5' GCTGCCATGGTTACAGAATACGAGAAACC) primers that were designed to introduce *Nco* I restriction sites at both the 5' and 3' ends. The PCR amplified cDNA was initially cloned into pSBS3062 at the *Nco* I restriction site that enabled placement of *AtDWF4* between the oleosin promoter and terminator. The expression cassette containing the oleosin promoter, *AtDWF4* coding region and the oleosin terminator, was excised with *Pst* I and cloned into the multiple cloning site of the binary vector pCAMBIA 2301 (CAMBIA, Canberra, Australia) to produce pCAMBIA-oleosin::*AtDWF4*. The latter was introduced into the *Agrobacterium* strain GV3101 by electroporation following sequence verification of the cloned DNA. The *Agrobacterium* culture was used to transform plants according to Clough and Bent (1998). Seeds collected from transformed plants ($T_0$) were selected on 30 mg $L^{-1}$ kanamycin. Homozygous lines were obtained by screening $T_2$ seeds for kanamycin resistance. Transgenic vector controls (VC) were generated similarly using the empty pCAMBIA2301vector for transformation.

### *5.2.3 Genomic DNA isolation and PCR analysis*

Genomic DNA was isolated from transgenic and WT Arabidopsis seedlings as described by Doyle and Doyle (1990). The genomic DNA was subjected to PCR analysis for identification of transgenic lines containing the desired insert by using oleosin-specific sequence as forward primer (5' AGCGGCTGCATGGTGACGC) and *AtDWF4* cDNA-specific sequence as reverse primer (5' GAATCAAGAACAAACAAAGTATG). PCR was performed using about 100 ng of DNA with an initial denaturation at $94^0$C for 4 min, followed by 30 cycles of denaturation (30 s at $94^0$C), annealing (45 s at $56^0$C), and extension (1 min at $72^0$C). After the last cycle, a final extension was carried out for 5 min at $72^0$C.

### *5.2.4 RNA isolation and RT-PCR analysis*

Total RNA was isolated from frozen plant tissue using SV Total RNA Isolation System (Promega, Madison, WI). For RT-PCR analysis, 5 µg of total RNA isolated from transgenic and WT Arabidopsis seedlings was reverse transcribed using the oligo (dT)18 primer and SuperScriptTM First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). The *AtDWF4* transcript abundance was analyzed by using the *NCOI-DWF4-F* and *NCOI-DWF4-R* primers that were used for cloning *AtDWF4* cDNA into pSBS3062 plasmid at the *Nco* I restriction site (section 5.2.2). PCR was carried out with an initial denaturation step of $94^0$C for 4 min, followed by 32 cycles for *AtDWF4* and 21 cycles for *ACTIN* (internal control) of denaturation (30 s at $94^0$C), annealing (45 s at $56^0$C), and extension (1 min at $72^0$C). After the last cycle, a final extension was carried out for 5 min at $72^0$C. For quantitative RT-PCR (qRT-PCR) analysis of *COR15A*, primer pairs *COR15A-F* (5' AAAGCTGCGGCGTATGTGGAG) and *COR15A-R* (5' CCTGCTTTACCCTCCGCGAAC) were used. PCR reactions were performed using SYBR-Green I (Invitrogen, Carlsbad, CA) at 0.1 x concentration, and Rotor Gene-3000 thermal cycler (Corbett Research, Sydney, Australia). Reactions were performed with an initial denaturation step at $94^0$C for 4 min followed by various cycles of denaturation (15 s at $94^0$C), annealing (30 s at $56^0$C) and extension (30 s at $72^0$C and 15 s at $83^0$C). The

following primers were used for *ACTIN* and *UBIQUITIN10 (UBQ10)* that served as internal controls in RT-PCR and qRT-PCR, respectively.

*ACTIN-F*: 5' TGCTCTTCCTCATGCTAT,

*ACTIN-R*: 5' ATCCTCCGATCCAGACACTG,

*UBQ10-F:* 5' CAGAACTTTGGCCGACTAC,

*UBQ10-R:* 5' ATGGTCTTTCCGGTGAGAG,

***5.2.5 Cold stress treatment***

Four independent homozygous transgenic lines expressing *AtDWF4*, (OD1, OD10, OD11 and OD12), two vector controls (VC2 and VC4) and WT were analyzed for tolerance to cold stress. Seeds were surface sterilized and plated on 1X Murashige and Skoog (MS) medium (Sigma, St. Louis) supplemented with B5 vitamins, 1% phytablend (Caisson labs, USA) and 1% sucrose. The plates were kept in the dark at $4^{0}$C for 3 days to encourage synchronized germination. Following this, the plates were transferred to a growth chamber and seeds were grown at $4^{0}$C for 10 days, followed by growth at $8^{0}$C for another 15 days. Plants were allowed to recover from cold stress at $22^{0}$C for 10 days. During stress and recovery periods, an 8/16 h (day/night) photoperiod and a light intensity of 80 $\mu$E $m^{-2}$ $s^{-1}$ was maintained. Percent survival was calculated by counting the number of seedlings that showed green leaves and no bleaching at 10 days after recovery. Values in Figure 5.3B are average percentages of data obtained in three biological replicates.

***5.2.6 Inhibition of germination by ABA***

Seeds were surface sterilized and plated on 1X MS supplemented with B5 vitamins, 1% phytablend (Caisson labs, USA) and 1% sucrose. ABA treatment was given by including 1.5 $\mu$M ABA in the medium, while the control plates contained 0.01% ethanol. The plates were first kept in the dark at $4^{0}$C for 3 days to encourage synchronized germination. Later the plates were transferred to a growth chamber set at $22^{0}$C to allow germination. Percent germination was determined 5 days after transferring plates to $22^{0}$C. Seeds with emerging cotyledons were scored as germinated. Values in Figure 5.4B are average percentages of data obtained in three biological replicates.

## 5.3 RESULTS

### *5.3.1 Genetic transformation and molecular characterization of transgenic plants*

Since ectopic and green tissue-specific overexpression of *AtDWF4/CYP* led to substantial increases in seed yield in Arabidopsis and rice, respectively, (Table 1; Choe et al., 2001; Wu et al., 2008), we wished to see if seed-specific overexpression of *AtDWF4* in Arabidopsis would positively impact seed development, germination and stress tolerance. A construct containing *AtDWF4* coding sequence under the control of the seed-specific oleosin promoter (Figure 5.1) was introduced into the genome of Arabidopsis via *Agrobacterium*. Seventy two transgenic plants were obtained, and 17 homozygous lines from these transgenic plants were selected in $T_2$ generation. To determine the presence of the oleosin::*AtDWF4* insertion in the genome of transgenic plants, PCR analysis was carried out on genomic DNA of homozygous $T_2$ plants. Ten transgenic lines (OD1, OD8, OD9, OD10, OD11, OD12, OD14, OD15, OD16 and OD17), two vector controls (VCs) and WT were screened for the presence of the oleosin::*AtDWF4* insert. As would be expected, no amplification was observed for WT and VC plants, while 7 out of 10 transgenic lines (OD1, OD10, OD11, OD12, OD14, OD15 and OD17) showed the presence of the insert (Figure 5.2A). Lines OD1, OD10, OD11 and OD12 yielded larger quantity of seeds as compared with other lines and were therefore used in further studies. The levels of *AtDWF4* transcripts were monitored by RT-PCR using cDNA generated from 5-day-old transgenic, VC2 and WT seedlings. Lines OD10 and OD12 clearly accumulated higher levels of *DWF4* transcripts relative to WT and VC2, while OD1 and OD11 showed only a minor increase in transcript levels (Figure 5.2B).

### *5.3.2 Seed-specific overexpression of AtDWF4 leads to enhanced cold tolerance*

BR effects on plant's ability to cope with high and low temperatures have been evaluated in several studies. Positive consequences of BRs in combating chilling stress were reported in maize, cucumber, tomato and rice (He et al., 1991; Katsumi, 1991; Kamuro and Takatsuto, 1991). To assess if seeds of transgenic lines overexpressing *AtDWF4* could tolerate cold temperatures better than the control group (WT and VC)

**Figure 5.1** Schematic of strategy employed for cloning of *AtDWF4*. **A)** cDNA sequence of *AtDWF4*. **B)** *AtDWF4* cloned into pSBS3062 containing oleosin promoter (oleosin P) and oleosin terminator (oleosin T). **C)** The pCAMBIA2301 plasmid carrying the oleosin::*AtDWF4* fusion at the *Pst* I restriction site. LB, left border; RB, right border; *KanR*, kanamycin resistance gene; 35SP, Cauliflower mosaic virus 35S promoter; 35ST, Cauliflower mosaic virus 35S terminator; NosT, Noplaine synthase terminator; Gus, β-glucoronidase gene. This plasmid was introduced into the genome of Arabidopsis ecotype Columbia via *Agrobacterium*-mediated transformation.

A

B

C

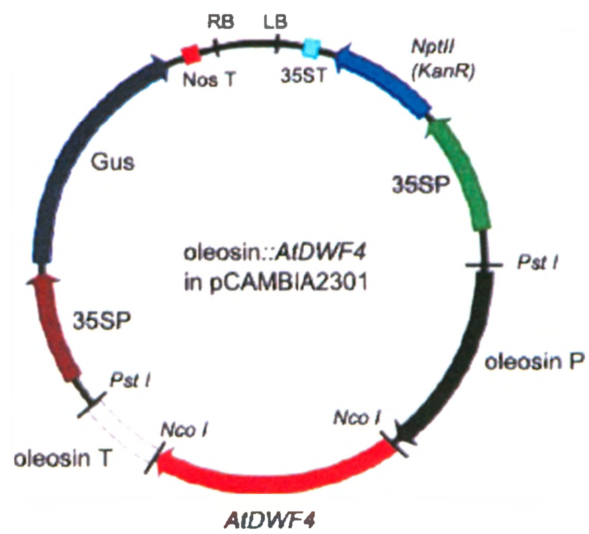

**Figure 5.2** Characterization of transgenic lines for the presence and expression of the transgene. **A)** Genomic DNA PCR showing the presence of oleosin::*AtDWF4* sequences in the genome of transgenic plants. PCR amplifications were performed on the genomic DNA of wild-type (WT), vector controls (VC2 and VC4), and ten transgenic lines (OD) using an oleosin-specific forward primer and *AtDWF4*-specific reverse primer. Seven (OD1, OD10, OD15, OD17, OD11, OD12 and OD14) out of the ten lines showed clear amplification of the expected size fragment, indicating the presence of transgene. **B)** RT-PCR analysis showing higher *AtDWF4* transcript levels in transgenic lines (OD1, OD10, OD11, and OD12) as compared to VC2 and WT. *ACTIN* was included as a control for constitutive expression.

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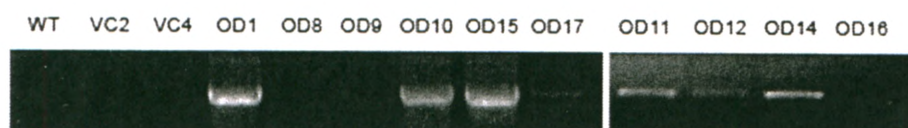


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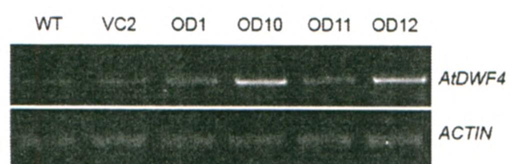


both during germination and growth, seeds of OD1, OD10, OD11, OD12, two VCs (VC2 and VC4) and WT were allowed to germinate at $4^0$C. No significant differences were seen between transgenic lines and the control group in seed germination percentages, but prolonged growth at $4^0$C led to cotyledons of all plants to turn purple-red in color, which indicates severe stress. To alleviate some of this stress, the temperature was changed to $8^0$C after initial growth for 10 days at $4^0$C. No visible morphological differences were observed between the different genotypes after 15 days under these conditions; all plants showed slight bleaching of leaves. However, when the seedlings were allowed to recover for 10 days at $22^0$C, the transgenic lines remained green and healthy while majority of the control group seedlings become bleached and stopped growing (Figure 5.3A). In line with these phenotypes, percentages of survival calculated as the number of green and unbleached seedlings were 63.5%, 83.5%, 64% and 62% for OD1, OD10, OD11 and OD12, respectively, and only 36.5%, 34.5% and 32.5% for VC2, VC4 and WT respectively. These results demonstrate that transgenic lines overexpressing *AtDWF4* have greater tolerance to cold temperatures during germination and early growth as compared to the control group.

### *5.3.3 Enhanced accumulation of cold-responsive COR15A transcripts in transgenic lines*

Cold stress can induce profound changes in the plant transcriptome. In Arabidopsis, cold stress results in the induction of CBFs/DREB1s (C-repeat binding factors or dehydration-responsive element-binding protein 1s), which can bind to *cis*-elements in the promoters of *COR (COLD-REGULATED)* genes and activate their expression (Stockinger et al., 1997). Recently it was shown that even moderate decreases in temperature could induce the expression of *COR15A* (*COLD-REGULATED 15A*) through the CBF signaling cascade and enhance freezing tolerance in Arabidopsis (Wang and Hua, 2009). To see if the higher cold tolerance of transgenic plants could be correlated to higher expression of at least one cold stress marker gene, transcript accumulation of *COR15A* was analyzed by qRT-PCR in different genotypes (Figure 5.3C). An approximate 2-fold increase in *COR15A* transcript levels in leaf tissue of transgenic lines OD1 and OD10 was seen relative to WT and VC2. Slightly lower

increases were observed for OD11 (1.5-fold) and OD12 (1.3-fold). Higher transcript levels of *COR15A* in transgenic lines suggest the possibility for additional members of the cold stress response gene regulon to also be expressed at higher levels in the transgenic lines, leading to greater cold tolerance in these plants. The study of additional such genes will provide a better understanding of how BR increases tolerance to cold stress.

### *5.3.4 Overexpression of AtDWF4 in Arabidopsis seed helps to overcome inhibition of germination by ABA*

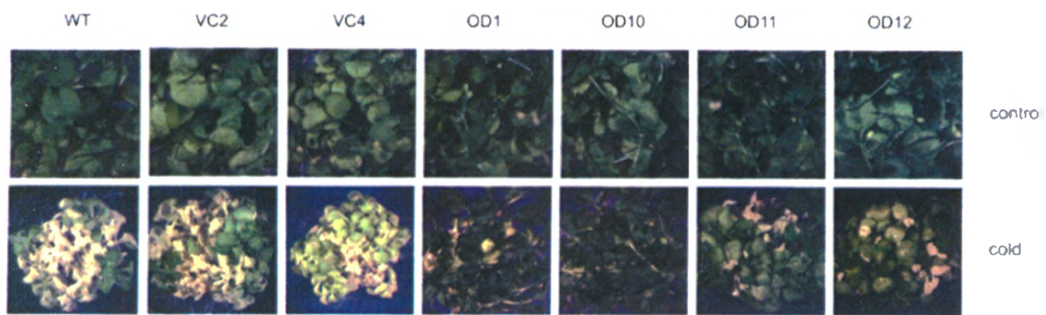
Several studies have pinpointed an antagonistic relationship between BR and ABA during seed germination, and BR has been shown to reverse the inhibition of germination by ABA (Steber and Mc Court, 2001). Assuming that overexpression of *AtDWF4* in seeds leads to increased BR levels in seeds, the germination rates of transgenic lines in the presence of 1.5 μM ABA were compared with those of control group. In the absence of exogenous ABA there were no differences in the germination rates of different genotypes (Figure 5.4A). However, in the presence of ABA, transgenic plants showed better germination efficiency as compared to WT and VCs (Figure 5.4A and B). Under these conditions, WT, VC2 and VC4 had germination rates of 26%, 35% and 48%, respectively. By contrast, transgenic lines OD1, OD10, OD11 and OD12 had germination rates of 55%, 80%, 90% and 91%, respectively. These results indicate that overexpression of BRs in seed can help to overcome inhibition of germination by ABA.

## 5.4 DISCUSSION

The endogenous levels of BRs are quite low and concentrations vary with plant species and developmental stage (Khripach et al., 2000). Pollen and immature seeds are the richest sources of BRs with levels ranging between 1-100 ng $g^{-1}$ fresh weight. Shoots and leaves usually have lower amounts in the range of 0.01-0.1 ng $g^{-1}$ fresh weight (Fujioka, 1998; Bajguz and Tretyn, 2003). In Arabidopsis, the highest levels of endogenous BRs and the highest expression of BR-related genes were detected in apical shoots followed by siliques, which contain actively developing embryos and seeds

**Figure 5.3** Effect of seed-specific overexpression of *AtDWF4* on cold tolerance of Arabidopsis seedlings. **A)** WT, VC (VC1 and VC2) and transgenic lines (OD1, OD10, OD11 and OD12) were allowed to germinate under cold conditions ($4^0$C for 10 days), followed by $8^0$C for another 15days. Later, the plants were allowed to recover from cold stress at $22^0$C for 10 days. Photographs were taken at the end of recovery period. **B)** Percent survival was calculated by counting the number of green and unbleached seedlings at the end of the recovery period. Data shown are average of three replicates. Error bars represent standard error (SE) of mean for three replicates. The threshold of significance as analyzed by student's t-test is indicated above the histograms ($*p \leq 0.03$). **C)** qRT-PCR analysis of *COR15A* in WT, VC and transgenic lines. RNA was isolated from the leaf tissue of seedlings collected at the end of growth at $8^0$C.

A

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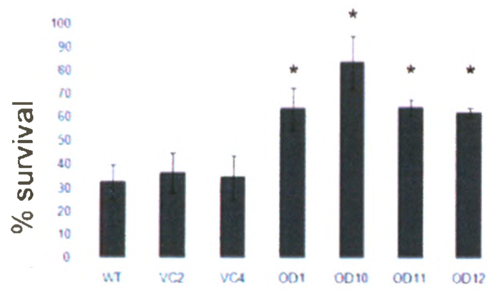


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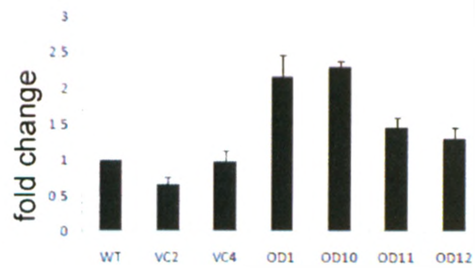

**Figure 5.4** Effect of seed-specific overexpression of *AtDWF4* on ABA-induced inhibition of germination. **A)** WT, VC (VC1 and VC2) and transgenic lines (OD1, OD10, OD11 and OD12) germinated in the presence or absence of 1.5μM ABA. **B)** Percent germination was determined after incubation of plates for 5 days at $22^{0}$C. Seeds with emerging cotyledons were scored as germinated. Data shown are average of three replicates. Error bars represent standard error (SE) of mean for three replicates. The threshold of significance as analyzed by student's t-test is indicated above the histograms (*$p \leq 0.01$).

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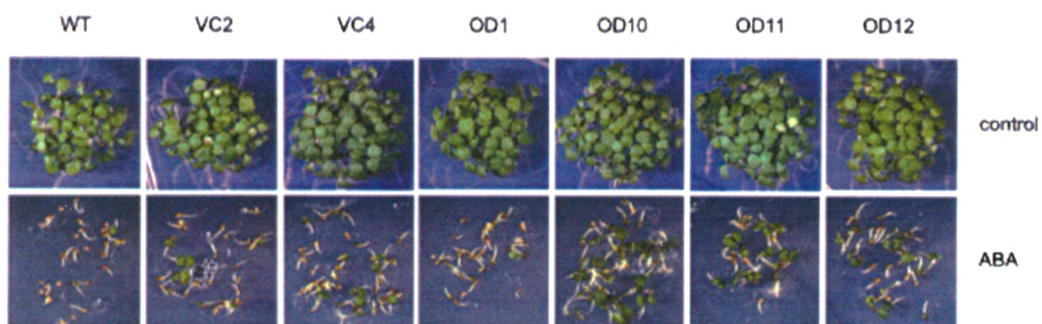


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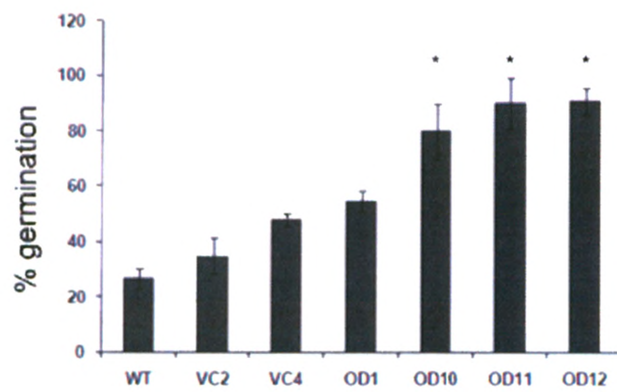

(Shimada et al., 2003). Despite our knowledge of the relatively higher levels of BRs in embryos and seeds, little has been done towards understanding the role of BRs in embryo development and seed germination. It has, however, been noted that BR-deficient Arabidopsis (*dwarf5*) and pea (*lk*) mutants have aberrations in seed shape (Choe et al., 2000; Nomura et al., 2004), and due to physical restriction of pods, seed size is reduced in BR-deficient Faba bean (*Vicia faba*) (Fukuta et al., 2006). Exogenous BR promotes seed germination and could rescue the retarded germination phenotype of biosynthetic and insensitive mutants of gibberellin, which is essential for normal seed germination (Steber and McCourt, 2001). Similarly, exogenous BR could rescue salt-induced inhibition of seed germination in *B. napus* (Kagale et al., 2007).

BRs elicit a wide spectrum of physiological and molecular responses involved in growth, development and stress tolerance (Khripach et al., 2000; Krishna, 2003; Divi and Krishna, 2009b). The growth-promoting effects of BRs under both normal and stress conditions have been studied at the molecular level, but the effects of BR on seed development and germination have been noted only in few preliminary studies. Since modulation of endogenous BR levels and BR response through changes in BR biosynthesis and perception genes has resulted in positive effects on growth, metabolism and seed yield (Table 1; Divi and Krishna 2009a), we embarked on determining the effects of overexrpessing *DWF4* in Arabidopsis seeds. While many aspects of the resultant transgenic material are remaining to be described, preliminary studies on cold stress tolerance in young seedlings and inhibition of germination by ABA indicate that a possible increase of BR levels in seeds increases cold tolerance, as well as germination efficiency in the presence of ABA. These results are novel in that a demonstration of increase in cold tolerance has previously not been attempted through modulation of endogenous BR levels, even though exogenous BR has been shown to increase cold tolerance in several studies. The present study can be strengthened by additional experiments in the following directions: The assumption that overexpression of *DWF4* in seeds increases BR levels needs to be verified by actual quantification of BR levels, and the cold stress conditions need to be better defined. It is clear that $4^0$C as the growth temperature was too cold to see any BR effects on a macroscopic level. The purple-red color of the cotyledons was likely due to accumulation of anthocyanins, which is a stress

response and usually precedes chlorophyll breakdown (Chalker-Scott and Scott, 2004; Feild et al., 2001). An increase in temperature to $8^0$C reduced such symptoms, but again produced no visible differences between the transgenic and control group plants. However, when cold stressed plants were allowed to recover at $22^0$C, a clear cut difference in the survival of transgenic plants was seen as compared to the control group (Figure 5.3). These conditions and results could be correlated with exposure of plants to fluctuating temperatures in the field and their differing abilities to override stress and resume growth when conditions become favourable. Our results indicate the potential of BR to impart some degree of advantage to seedlings towards cold conditions, but do not provide any range of temperature conditions over which the transgenic plants can perform better than WT. In the future, temperatures ranging from 8-$15^0$C should be tested for plant growth under sub-optimal conditions and recovery from it.

Several cold-inducible genes have been identified and characterized in plants (Chinnusamy et al., 2007). Previously we have shown that exogenous treatment with EBR induces the expression of several stress-responsive genes, including those involved in cold tolerance (Kagale et al., 2007; Chapter 4). A correlation of *COR15A* transcript levels with cold tolerance is well established (Lin and Thomashow, 1992; Wang and Hua, 2009). Constitutive expression of *AtCOR15A* in Arabidopsis significantly increased survival of isolated protoplasts frozen over -4.5 to -7°C (Steponkus et al., 1998). The approximate 2-fold up-regulation of *COR15A* in transgenic seedlings as compared to the control group is indicative of BR's effect on at least one cold-regulated gene. Interestingly, Arabidopsis seedlings grown on exogenously supplied EBR also had increased levels of *COR15A* transcripts (upto 5-fold) under both no-stress and heat stress conditions (Chapter 4). Thus, both exogenous BR treatment and possible endogenous overproduction of BR lead to higher expression of *COR15A* as compared to control plants. Based on these results we extrapolate that other cold-regulated genes may also be up-regulated by the possible overproduction of BRs in seeds, which may be the underlying cause, at least in part, for the better ability of trangenics to recover from cold stress. More detailed studies on gene expression in transgenic plants will provide insights into how these seedlings can recover from stress more efficiently than the control group. Of the transgenic lines tested, OD10 had the greatest expression of *DWF4* and *COR15A*

trasncripts and the highest level of survival following cold stress. Such a correlation was not found with other transgenic lines and may involve protein levels or other factors contributing to it.

ABA plays important roles in seed maturation and dormancy as well as in adaptation to environmental stresses (Finkelstein et al., 2008; Shinozaki and Yamaguchi-Shinozaki, 2000). BR and ABA interact antagonistically in some aspects of stress responses and seed germination (Zhang et al., 2009; Steber and McCourt, 2001). For example, the BR-deficient mutant *det2-1*, and the BR-insensitive mutant *bri1-1*, germinate well under normal growth conditions, but are more sensitive to inhibition of germination by ABA than WT (Steber and McCourt, 2001). With regards to stress responses, it is demonstrated in chapter 3 that ABA suppresses BR-induced thermotolerance in Arabidopsis. The increased germination efficiency of oleosin::*AtDWF4* seeds in the presence of ABA, as compared to control seeds (Figure 5.4), confirms the ability of BR to suppress ABA-induced inhibition of germination. It should be noted that the Arabidopsis oleosin promoter is inducible by ABA (Plant et al., 1994). Thus, it is possible that ABA treatment further enhanced the expression of oleosin::*AtDWF4,* resulting in even higher levels of endogenous BR and greater advantage in overriding inhibition of germination by ABA.

Although seedlings grown on exogenously supplied BR are resistant to heat and salt (Kagale et al., 2007), preliminary experiments involving heat and salt stress did not result in any noticeable differences between the transgenic and control group plants. Since younger seedlings (5-day-old) were employed in these experiments as opposed to 21-day-old seedlings used in exogenous treatment, it is possible that the BR effect on these stresses was missed. The stress assays need additional standardization to capture BR's effect, if any, on these stresses.

Oilbodies are the storage organelles in plants for lipids that accumulate as TAGs (Murphy, 1993). Oleosins cover the oilbodies and are important for controlling oilbody structure and lipid accumulation (Huang, 1992; Siloto et al., 2006). Exogenous BR induces the expression of several genes involved in protein synthesis, lipid metabolism and embryo development (Chapter 4). Effect of BR in promoting protein synthesis by modulating the expression of some of the components of the translational machinery has

also been demonstrated (Dhaubhadel et al., 2002). Recently it has been shown that BIM1, a bHLH protein involved in BR signaling, controls embryonic patterning in Arabidopsis (Chandler et al., 2008). In light of these observations it can be expected that BR affects TAG accumulation and formation of oilbodies. Future studies should investigate embryo development, seed oil content and quality in transgenics and compare it to WT. If higher oilbody accumulation is observed, the current strategy can be applied in molecular farming. The oleosin expression system is being used for transgenic production of therapeutic proteins like insulin and hirudin as oleosin fusions (Capuano et al., 2007; Nykiforuk et al., 2006). Increased oilbody content can lead to increased transgenic protein yield as more protein can be tagged and retained on the surface of oilbodies.

In summary, the present study provides genetic evidence for a role of BRs in promoting cold tolerance and germination during inhibitory conditions. Further analysis of the transgenic seed generated in this study will indicate whether or not the current strategy has potential for commercial applications in seed oil industry and molecular farming.

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# CHAPTER 6

# GENERAL DISCUSSION AND CONCLUSIONS

# CHAPTER 6

## 6.1 GENERAL DISCUSSION

Brassinosteroids (BRs) are a group of steroidal plant hormones that are essential for normal plant growth and development (Clouse and Sasse, 1998). Another remarkable feature of BRs is their ability to increase tolerance in plants against a wide spectrum of abiotic and biotic stresses (Krishna, 2003), but a good understanding of the molecular mechanisms underlying this phenomenon is lacking. The present study is an investigation at the molecular level to explore the mechanisms by which BRs increase stress tolerance.

Though BRs have been implicated in drought, cold and salt stress responses (Krishna, 2003), experimental conditions under which the stress alleviating effects of BR can be studied in a reproducible manner at the morphological level have not been described in literature nor have there been any molecular studies in this direction. Thus, there is a lacuna of convincing evidence for the ability of BR in modulating plant responses to a variety of environmental stresses. Our previous studies have established the role of 24-epibrassinloide (EBR), a BR, in modulating plant response to high temperature stress (Dhaubhadel et al., 1999; 2002). The present study established conditions to study the effects of EBR on drought stress responses in Arabidopsis in a reproducible manner and also provides the first molecular evidence for a role of BR in plant responses to drought stress. The effect of EBR in increasing drought tolerance in Arabidopsis seedlings was evident both from the reduction in visible morphological symptoms of drought stress and increased expression of known drought and cold stress responsive genes (Chapter 2; Kagale et al., 2007). Although BR-induced changes in transcript levels are modest, BR-induced changes in plant phenotypes are clear, implying that the changes in gene expression by BR are sufficient for inducing the phenotypes. Thus, the results of the present study along with those published by Kagale et al. (2007), demonstrate that BR enhances tolerance of Arabidopsis seedlings to a variety of abiotic stresses, and that this effect involves changes in the expression of genes encoding both structural and regulatory proteins.

In order to address the question of how BR mediates tolerance to various stresses, it is reasonable to assume that physiological and biochemical alterations resulting from BR-mediated gene expression changes might contribute to enhanced stress tolerance. A survey of genes responsive to BR under controlled stress conditions can shed light on mechanisms underlying BR mediated stress tolerance. Experimental conditions to study positive effects of BR on heat stress (HS) tolerance of Arabidopsis in a reproducible manner were established previously (Kagale et al., 2007). In the present study, analysis of global gene expression was undertaken using Affymetrix ATH1 genome arrays in BR-treated and untreated Arabidopsis seedlings before, during and after HS (Chapter 4). The transcriptional changes in response to EBR under no-stress (0 h) and HS (1 h and 3 h at $43^0$C); and transcriptional changes in response to HS (1 h and 3 h at $43^0$C) were analyzed. Investigation of the differentially expressed genes revealed that a major category of genes affected by BR consists of genes previously linked with abiotic and biotic stress tolerance. Several transcripts related to signal transduction and regulation of metabolism were significantly changed by BR treatment. These include protein kinases, phosphatases, calcium signaling genes, transcription factors and genes involved in redox regulation and hormone metabolism. Genes involved in sucrose-starch metabolism and secondary metabolism were also regulated by BR under no-stress and stress conditions. The expression patterns of these genes suggest that BR promotes accumulation of simple soluble sugars and various phenylpropanoid compounds like flavonoids, lignins and anthocyanins during stress conditions. Thus, it is clear that BR treated plants are better equipped to regulate these processes by modulating the expression of related genes thereby enabling the structural and physiological adjustments required to sustain in adverse environmental conditions.

Several similarities with previous BR response microarray studies were observed in this study. For example, down-regulation of type A response regulators (ARRs) was commonly observed in all BR genomic studies (Vert et al., 2005), in our study *ARR4, ARR7, ARR15* and *ARR16* were found to be down-regulated by BR. Plentiful connections with other plant hormones were also observed; several genes involved in the biosynthesis and/or signaling of other plant hormones were responsive to BR. *In silico* analysis of the 'BR response genes', using publicly available microarray datasets, revealed considerable

overlap with genes responsive to various abiotic stresses and other plant hormones, especially ABA and JA. Further, we identified several novel genes (previously uncharacterized) and genes that are maximally under BR influence as compared to other hormones. Taken together, our data indicate that treatment with BR induces the expression of several stress response genes even in the absence of stress and during stress augments these responses thereby conferring stress tolerance. In mediating the stress responses there appears to be a complex interaction of BR with other hormone pathways.

Morphological and physiological characterization of knockout (KO) mutants of a few genes identified in the microarray screen has revealed new stress-related BR response genes in Arabidopsis. In addition, a novel gene, maximally up-regulated by BR, *BRRLK* was identified in the current study. A role for *BRRLK* in heat and salt stress tolerance was established by T-DNA mutant analysis. Phylogenetic analysis of BRRLK revealed similarities with proteins involved in pathogen defense and cell-death responses. Further experiments using KO mutants and overexpressors of *BRRLK* will help clarify its role in plant defense. Thus, the results of current study have shed light on a macro scale on how BR may promote stress tolerance in plants.

Since a subset of genes identified in the microarray screen is related to biosynthesis and signaling of other plant hormones, and the effects of BR on stress tolerance are most pronounced when seedlings are grown in the presence of EBR for 21 days (long-term treatment) (Kagale et al., 2007), we postulated the involvement of other phytohormones in this process. Several hormone pathways, such as of abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and salicylic acid (SA), have been linked with one or more environmental stresses, including heat stress (HS). Using a collection of Arabidopsis mutants that are either deficient in or insensitive to ABA, ET, JA and SA, we studied the effects of EBR on basic thermotolerance and salt tolerance of these mutants (Chapter 3). The results of this study showed that a redox-sensitive protein and a master regulator of SA-responses, NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1) is required for EBR mediated increase in thermotolerance and salt tolerance, but not for EBR mediated induction of SA-marker gene, *PR-1* (*PATHOGENESIS RELATED1*). With the exception of SA-insensitive *npr1-1* mutant, the positive impact of EBR on thermotolerance was evident in all mutants studied. Further, it was observed that

the effect of EBR was more pronounced in the ABA deficient *aba1-1* mutant, indicating that endogenous ABA levels mask BR effects in wild-type (WT) seedlings. The positive effect of EBR on inhibition of germination by salt is previously known (Kagale et al., 2007). In the present study it was found that EBR could rescue the ET-insensitive *ein2* mutant from its hypersensitivity to salt stress-induced inhibition of seed germination, but remained ineffective in increasing the survival of ET-overproducer *eto1-1* and SA-insensitive *npr1-1* seedlings on salt. It was also found that EBR can induce the expression of other hormone marker genes in the corresponding mutant backgrounds. Thus, from the collection of mutants used here it would appear that BR exerts anti-stress effects both independently as well as through interactions with ABA, ET, JA and SA. The dependency of BR on NPR1 in mediating stress tolerance is a first time observation made in this study. Future studies directed at global gene expression analysis in WT and *npr1* mutant in response to HS and BR as separate and combined treatments should indicate NPR1-dependent molecular changes and help clarify the role of NPR1 in BR-mediated increase in thermotolerance.

Since BRs control several important agronomic traits such as flowering time, plant architecture, seed yield, and stress tolerance, the genetic manipulation of BR biosynthesis or signaling offers a unique possibility of significantly increasing crop yields. Several studies have demonstrated the effects of increasing endogenous BR levels on plant growth and yield, while very few studies were available showing the stress tolerance effects of endogenous BR overproduction (Divi and Krishna, 2009). Preliminary studies in Arabidopsis have indicated that an increase in endogenous levels of BRs through overexpression of *AtDWF4*, a gene encoding an enzyme involved in BR biosynthesis, can lead to increases in vegetative growth and seed yield (Choe et al., 2001). In order to assess the impact of overproducing BRs on seed development, germination and stress tolerance, the *AtDWF4* gene was overexpressed under the control of a seed-specific oleosin promoter. Our preliminary analysis showed that the resulting transgenic plants had increased cold tolerance and enhanced tolerance to inhibition of germination by ABA (Chapter 5). This is the first time a correlation can be made between a possible increase in endogenous BR levels and cold stress tolerance in plants. However, the assumption that overexpression of *AtDWF4* in seeds will increase BR levels needs to

be verified by actual quantification of BR levels. Thus, the study provides genetic evidences for the role of BRs in promoting tolerance against cold stress and germination during inhibitory conditions. The transgenic seed generated in this study may turn out to be a genetic resource with potential commercial applications in seed oil industry and molecular farming.

In summary, the combined results of the present study confirm the ability of BR to confer tolerance to a range of stresses. The global gene expression analysis provided insights into molecular mechanisms by which BR imparts stress tolerance and lead to identification of novel BR response genes and their abiotic stress-related functions were revealed. For the first time convincing evidences were obtained for the cross-talk of BR with other plant hormones in mediating stress responses. Genetic evidences for the role of BRs in promoting stress tolerance were obtained and genetic resources that will enable further studies to explore the feasibility of BRs for commercial applications were generated.

## 6.2 FUTURE WORK

Genome wide transcript analysis of BR-treated seedlings has allowed identification of several BR response genes with putative roles in stress tolerance. The stress-related functions of a handful of these genes were confirmed by KO mutant analysis. Future studies will be directed towards characterizing more BR response genes and pinpointing the regulatory genes that might have a multitude effect on BR-regulated processes. Detailed functional analysis of genes encoding regulatory proteins like transcription factors, protein kinases, phosphatases and other signaling components will help to integrate different signaling pathways and diverse physiological processes that together constitute the BR-directed increase in stress tolerance.

Changes in gene transcription have been thought to be the major mode of action by phytohormones, but post-transcriptional changes such as changes in transcript and protein stability, and protein synthesis are also possibilities that need to be studied in this context. The effect of BR on protein synthesis during heat stress was clearly demonstrated in *B. napus* (Dhaubhdel et al., 2002). A recent proteomic study for understanding BR responses identified 42 BR-regulated proteins in Arabidopsis, majority

of which were not identified by earlier microarray screens (Deng et al., 2007). Since BR-induced changes in transcript levels are modest, it is possible that BR produces more substantial changes at the protein level. Thus, proteomics analysis of BR-mediated stress responses will certainly expand our understanding of how BR affects stress tolerance. Alongside unraveling the BR mode of action, other aspects such as uptake, transport and stability of BRs should continue to be explored.

Several BR response genes were related to pathogen resistance. The available T-DNA mutants of BR response genes can be tested for pathogen resistance. Moreover, the study highlighted the possible interactions of BR with other plant hormones and critical roles for NPR1 and WRKY70 have been suggested. While these results provide the initial clues, future studies involving *WRKY70* knock out mutants, physical interactions of transcripton factors like BES1 or BZR1 with other hormone marker genes might further unravel the mode of BR cross-talk with other plant hormones.

Despite an antagonistic relationship between BR and ABA in mediating HS responses (Chapter 3), and during seed germination (Chapter 5); the data obtained in microarray study (Chapter 4) suggests that ABA levels rise in response to both BR and HS. Based on these results it can be speculated that BR augments ABA levels and ABA-related effects during HS and that BR effects become more apparent when ABA levels are compromised. This hypothesis can be tested by determining endogenous levels of ABA in BR-treated and untreated Arabidopsis plants, as well as in the transgenic plants overexpressing *AtDWF4*.

Seed-specific overexpression of *AtDWF4* in Arabidopsis resulted in increased tolerance to cold stress and enhanced germination on ABA. Further studies on BR overexpressing lines for their tolerance to other environmental stresses will strengthen the link between BRs and stress tolerance. Phenotypic analysis of embryo cells and analysis of seed oil content in the transgenic seed will provide insights into role of BRs in embryo development and seed oil accumulation which can be exploited for commercial applications.

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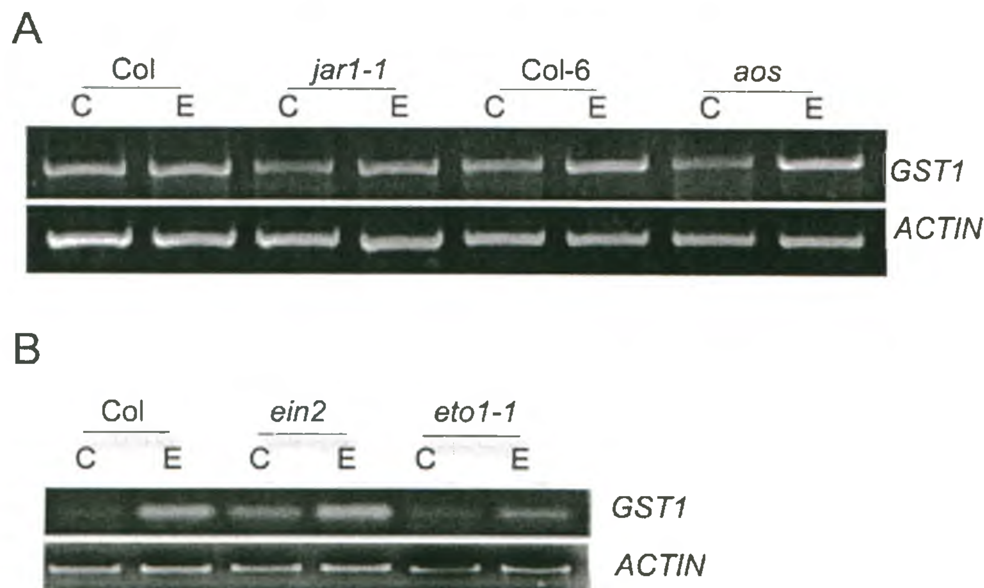
# APPENDICES




**Appendix 3.1** Effect of EBR on the expression of *GST1* in JA and ET mutants. Total RNA was isolated from wild-type (Col and Col-6) and mutant seedlings grown in the absence (C) or presence (E) of EBR at $22^0$C, and transcript levels were analyzed by RT-PCR. *ACTIN* was included as a control for constitutive expression. **A)** Expression profile of the stress-responsive *GST1* in WT and JA genotypes. **B)** Expression profile of the stress-responsive *GST1* in WT and ET genotypes

| AGI ID | Forward primer | Reverse primer |
|---|---|---|
| At3g56400 | 5' GGCAACAAAGCAAGTCCAGAAGG | 5' CTTGGGAGTTTCTGCGTTGGTTG |
| At3g04720 | 5' ACAAGGCCATCTCATTGTTG | 5' GATCAATGGCCGAAACAAG |
| At3g45140 | 5' CTCTTCAGAGCACGCTACG | 5' GAAGATGGAGGGAAGAGCTG |
| At1g19610 | 5' GGAAGAATTTGCGAGAGGAG | 5' GCACGTTCCCATCTCTTAC |
| At4g14365 | 5' GGCAACAACAATCACAGTCC | 5' CTCGAGTCCTGCTCCTTCATG |
| At3g13672 | 5' GACAGTCACAAGACGGTTCG | 5' AACTTCAGCTCCTTGTCGGTG |
| At5g13170 | 5' TTGTATTCCTCGCTCCAGTG | 5' AGCATGCAGCTAAATAGCG |
| At5g59310 | 5' CACCAACTGCGCCACCATCAAG | 5' GCCATCAAGACAAACAAAGAC |
| At1g21250 | 5' GAGTTACTTTGCGACTGCCA | 5' CAGCTTCCTGGATCTCCTTC |

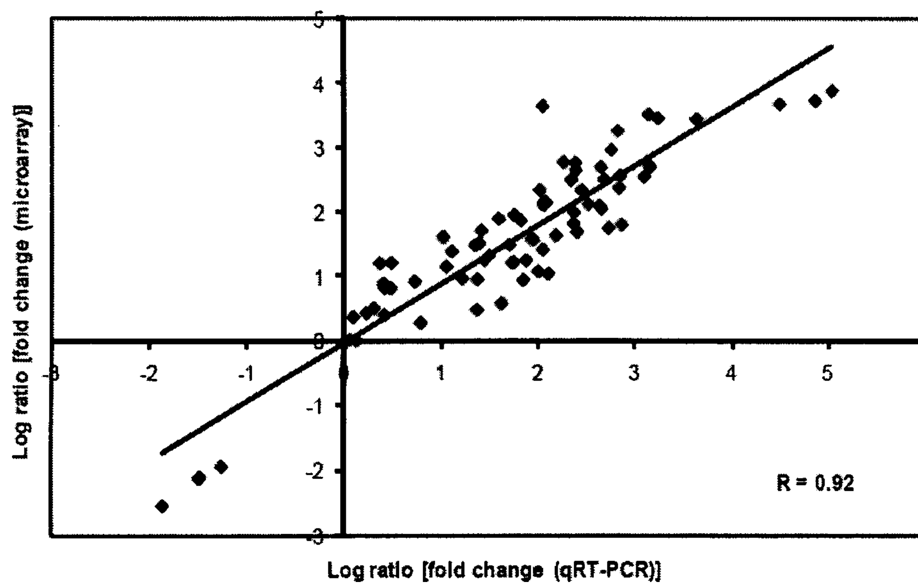
**Appendix 4.1** Primers used in quantitative RT-PCR (qRT-PCR) analysis

| Gene | Primer name | Sequence |
|---|---|---|
| *WRKY17* | *WRKY17-LP* | 5' TGGATTTTGGTTAAAGACCTTC |
| | *WRKY17-RP* | 5' AGCAAGAAAGATCGAAGAGCC |
| *WRKY33* | *WRKY33-LP* | 5' CTCTTTTTCTCGATTCCCCTG |
| | *WRKY33-RP* | 5' TTCAGGTTCACTCCCACAATC |
| *ACP5* | *ACP5-LP* | 5' ATTGTAATAACACCGGAGGGC |
| | *ACP5-RP* | 5' CTAAAACCCCCAAAGCTTTTG |
| *BRRLK* | *BRRLK-LP* | 5' CATCAGAAGGAGCAGGAACAG |
| | *BRRLK-RP* | 5' ACATCCGGGAGATTTGGTATC |

**Appendix 4.2** Primers used for confirming the homozygous insertion of the T-DNA lines

| Gene | Primer name | Sequence |
|---|---|---|
| *WRKY17* | *WRKY17-F* | 5' GTGACGTACGAAGGAGAGC |
| | *WRKY17-R* | 5' ACCAAACACCAAACCACTC |
| *WRKY33* | *WRKY 33-F* | 5' GCAAAGGAAAGGAGAGGATG |
| | *WRKY 33-R* | 5' GGTTTAGGATGGTTGTGGCT |
| *ACP5* | *ATACP5-F* | 5' CGAAGAGCTTCTTCCGATTC |
| | *ATACP5-R* | 5' TTTAGAAGTGACCCATTTGTGC |
| *BRRLK* | *BRRLK-F* | 5' ATTCGATCCAACACACCTC |
| | *BRRLK-R* | 5' CGTCGTTGGCTAAGAGTTTG |

**Appendix 4.3** Primers used for RT-PCR confirmation of the knockout lines




**Appendix 4.4** Correlation between microarray and qRT-PCR data. $Log_2$-tranformed fold change values (EBR-treated *vs.* untreated) for 20 genes obtained by microarray and qRT-PCR were compared at 0 h, 1 h , 3 h and 6 hR time points. The best-fit linear regression curve and the correlation coefficient (*r*) value are shown.